THE BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

VOLUME LXXIX

AUGUST TO DECEMBER, 1940

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THE MARINE BIOLOGICAL LABORATORY

FORTY-SECOND REPORT, FOR THE YEAR 1939—
FIFTY-SECOND YEAR

I. TRUSTEES

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Charles Packard, *Ex. Off.*
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Laurence Irving, to serve until 1940.
S. O. Mast, to serve until 1940.

L. V. Heilbrunn, to serve until 1941.
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C. C. Speidel.

The Evening Lecture Committee

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M. H. Jacobs.
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II. ACT OF INCORPORATION

No. 3170

Commonwealth of Massachusetts

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, Henry B. Pierce, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an

existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 11.30 A.M., daylight saving time, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years. There shall be thirty-two Trustees thus chosen divided into four classes, each to serve four years, and in addition there shall be two groups of Trustees as follows: (*a*) Trustees *ex officio,* who shall be the President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer and the Clerk; (*b*) Trustees Emeritus, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next annual meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee Emeritus for life. The Trustees *ex officio* and Emeritus shall have all rights of the Trustees except that Trustees Emeritus shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. Inasmuch as the time and place of the Annual Meeting of Members is fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of said meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and

define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1939.

The accounts have been audited by Messrs. Seamans, Stetson and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and is open to inspection by members of the Corporation.

At the end of the year 1939, the book value of the Endowment Fund in the hands of the Central Hanover Bank and Trust Company as Trustee, was

General Fund,	Securities (market $846,763.12)	$ 899,277.72
	Real Estate	24,860.00
	Cash	356.81
Library Fund,	Securities (market $148,086.70)	$ 161,767.17
	Real Estate	20,102.88
	Cash	12,538.80
		$1,118,903.38

The income collected from these Funds was as follows:

General Endowment	$35,471.86
Library	6,700.05
	$42,171.91

The income in arrears on these Funds at the end of the year was:

Arrears General Fund	$10,957.06
Arrears Library Fund	4,365.75
	$15,322.81
Arrears at the end of 1938	$16,968.86
an improvement of	$ 1,646.05

The dividends from the General Biological Supply House totalled $14,986.00, a slight increase over the year before.

Retirement Fund: A total of $4,060 was paid in pensions of which $726 was advanced from current funds. The book value of the securities and real estate in this Fund at the end of the year was .. $17,111.75
balance of cash 31.28
$17,143.03
of which 923.20

was due current funds for advances, leaving a net book value of $16,219.83

Plant Assets: The land (exclusive of the Gansett and Devil's Lane tracts) the buildings, equipment and library represent an investment of $1,812,054.80
less reserve for depreciation 539,462.84

or a net of $1,272,591.96

Income and Expenses: Income including a donation of stock by Dr. Frank R. Lillie valued at $10,500 exceeded expenses including depreciation of $25,013.41, by $11,176.50.

There was expended from current funds for plant account a net of $19,281.23 and in payment of mortgage and note indebtedness a total of $9,000.00.

At the end of the year the Laboratory owed $3,500 on a note, the last of the notes given for the purchase of the Bar Neck property, which

note has since been paid. It owed on Accounts Payable $3,993.22 against which it had accounts receivable of $9,585.34 and cash in bank of $14,790.37. The Laboratory now has no mortgage indebtedness.

In addition to the gift of $20,000 for Hurricane Damage from the Carnegie Corporation and a gift of 300 shares of Crane Company stock received from Dr. Frank R. Lillie, both received early in 1939 and referred to in my last report, the Laboratory received in 1939—

From anonymous donor toward purchase of Bay Shore Lot No. 6	$1,000.00	
From National Research Council toward purchase of X-Ray Tube	275.00	
From General Electric Company toward purchase of X-Ray Tube	150.00	
From General Electric Company, Vapor Lamp Division, Sodium Lamp	62.50	$1,487.50

and Dr. Oliver Strong made a contribution in the form of a sale of Bay Shore Lot No. 6 at a figure considerably below its market value.

Following is the balance sheet, the condensed statement of income and outgo, and the surplus account all as set out by the accountants.

EXHIBIT A

Marine Biological Laboratory Balance Sheet, December 31, 1939

Assets

Endowment Assets and Equities:			
Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee —Schedules I-a and I-b		$1,118,903.38	
Securities and Cash—Minor Funds—Schedule II		8,994.51	$1,127,897.89
Plant Assets:			
Land—Schedule IV	$ 111,425.38		
Buildings—Schedule IV	1,237,683.99		
Equipment—Schedule IV	174,335.50		
Library—Schedule IV	288,609.93	$1,812,054.80	
Less Reserve for Depreciation		539,462.84	
		$1,272,591.96	
Cash in Dormitory Building Fund		223.24	
Cash in Reserve Fund		24.65	$1,272,839.85

Brought forward				$2,400,737.74
Current Assets:				
Cash			$ 14,790.37	
Accounts and Notes-Receivable			9,585.34	
Inventories:				
Supply Department	$ 33,139.40			
Biological Bulletin	10,252.20		43,391.60	
Investments:				
Devil's Lane Property	$ 44,771.89			
Gansett Property	5,923.29			
Stock in General Biological Supply House, Inc.	12,700.00			
Other Investment Stocks	17,770.00			
Securities, Real Estate, and Cash —Retirement Fund List—Schedule V, viz.,				
Retirement Fund Portion	16,219.83			
Current Account Portion	923.20		98,308.21	
Prepaid Insurance			3,316.53	
Items in Suspense (Net)			210.95	$ 169,603.00
				$2,570,340.74

Liabilities

Endowment Funds:			
Endowment Funds—Schedule III	$1,117,824.52		
Reserve for Amortization of Bond Premiums	1,078.86	$1,118,903.38	
Minor Funds—Schedule III		8,994.51	$1,127,897.89
Plant Liabilities and Funds:			
Notes—Payable a/c Bar Neck Property Purchase		$ 3,500.00	
Donations and Gifts—Schedule III		1,039,890.11	
Other Investments in Plant from Gifts and Current Funds		229,449.74	$1,272,839.85
Current Liabilities and Surplus:			
Accounts—Payable		$ 3,993.22	
Reserve for Additional Repairs and Replacements on account of Hurricane Water-Damage		2,403.49	
Current Surplus—Exhibit C		163,206.29	$ 169,603.00

EXHIBIT B

Marine Biological Laboratory Income and Expense, Year Ended December 31, 1939

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		$ 35,471.86		$ 35,471.86
Library Fund		6,700.05		6,700.05
Donations		10,500.00		10,500.00

Instruction	$ 9,131.39	9,995.00		863.61
Research	6,147.59	16,210.00		10,062.41
Evening Lectures	202.15		$ 202.15	
Biological Bulletin and Membership Dues	8,621.47	9,158.18		536.71
Supply Department—Schedule VI	37,412.21	35,239.33	2,172.88	
Mess—Schedule VII	24,913.02	26,040.73		1,127.71
Dormitories—Schedule VIII	22,978.26	12,966.90	10,011.36	
(Interest and Depreciation charged to above 3 departments—See Schedules VI, VII, and VIII)	*23,826.40*			23,826.40
Dividends, General Biological Supply House, Inc.		14,986.00		14,986.00
Dividends, Crane Company		300.00		300.00
Rents:				
Bar Neck Property		3,581.69		3,581.69
Bay Shore Property	82.61		82.61	
Howes Property	432.41	480.00		47.59
Janitor House	80.22	360.00		279.78
Newman Cottage	71.34		71.34	
Danchakoff Cottages	497.60	750.00		252.40
Sale of Library Duplicates		98.84		98.84
Apparatus Rental		1,030.76		1,030.76
Interest on Notes—Receivable		60.00		60.00
Sundry Income		40.50		40.50
Maintenance of Plant:				
Buildings and Grounds	23,410.66		23,410.66	
Chemical and Special Apparatus Expense	14,540.59		14,540.59	
Library Expense	7,806.06		7,806.06	
Truck Expense	568.90		568.90	
Workmen's Compensation Insurance	460.18		460.18	
Sundry Expense	18.90		18.90	
General Expenses:				
Administration Expense	12,342.08		12,342.08	
Endowment Fund Trustee and Safe-keeping	1,013.81		1,013.81	
Interest on Notes and Mortgage —Payable	537.50		537.50	
Bad Debts	337.38		337.38	
Reserve for Depreciation	25,013.41		25,013.41	
	$172,793.34	$183,969.84	$98,589.81	$109,766.31
Excess of Income over Expense carried to Current Surplus—Exhibit C	11,176.50		11,176.50	
	$183,969.84		$109,766.31	

EXHIBIT C

Marine Biological Laboratory, Current Surplus Account, Year Ended December 31, 1939

Balance, January 1, 1939			$138,197.19
Add:			
Excess of Income over Expense for Year as shown in Exhibit B		$11,176.50	
Emergency Grant of Carnegie Corporation		20,000.00	
Donation on account of Hurricane Water Damage		10.00	
Excess of Marine Insurance over Estimated Loss		95.04	
Reserve for Depreciation Charged to Plant Funds		25,013.41	
Equipment Replacements Added to Plant		4,192.90	60,487.85
			$198,685.04
Deduct:			
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV,			
Land	$ 32.27		
Buildings	247.95		
Equipment	7,338.14		
Library	14,338.92	21,957.28	
Equipment Replacements		4,192.90	
		$26,150.18	
Less Received for Plant Assets Disposed of	2,676.05		
Loss on Equipment Charged off due to Hurricane Water-Damage	477.63	3,153.68	
		$22,996.50	
Payment on Plant Mortgage and Notes—Payable		$ 9,000.00	
Pensions Paid	4,060.00		
Less Retirement Fund Income	577.75	3,482.25	35,478.75
Balance, December 31, 1939—Exhibit A			$163,206.29

Respectfully submitted,
LAWRASON RIGGS, JR.,
Treasurer.

V. REPORT OF THE LIBRARIAN

Aside from the appropriation of $18,800 for the Library for 1939, which has not been changed for the past three years, the Executive Committee raised the yearly salary of one assistant by $50 and allowed $800 for extra service if necessary for the year 1939 only in order that the reprint collection might be completely checked. The annual sum

was apportioned at the beginning of the year as follows: books, $1000; serials, $6000; binding, $1500; express, $300; supplies, $500; salaries, $7200; back sets, $2350; total, $18,850. The appropriation was expended as follows: books, $236.88; current serials, $4749.84; binding, $2052.98; express, $122.18; supplies, $308.57; salaries, $7200; back sets, $4040.76; total, $18,711.21. Of the $800 allowed for extra help only $123.60 was spent and the remainder reverted to the Laboratory. During the year $98.84 was realized by the sale of duplicates and this sum will be retained by the Library for emergencies.

It is a source of satisfaction to be able to report (as of April, 1940) that practically all German journals and books have been received and paid for complete to the end of the year 1939.

The Woods Hole Oceanographic Institution appropriated $600 for the Library, the sum allowed by this Institution since 1936. This was expended to the amount of $525.91 and the account rendered to that Institution separately.

This year the Library received fewer current serials by 60 titles than in 1938 due chiefly to the Librarian's action in cutting off exchanges with the "Biological Bulletin" where the receipts from foreign countries had become completely inadequate or had actually ceased. This decline in number of exchanges will undoubtedly be enhanced during the next years. The current serials are now 1246 in number of which 430 are subscriptions, 390 (15 new) purchases of the Marine Biological Laboratory, 40 (1 new) of the Woods Hole Oceanographic Institution; 608 are exchanges, 536 (6 new) with the "Biological Bulletin"; and 72 (2 new) with the Woods Hole Oceanographic Institution publications; and 199 come as gifts to the former and 9 as gifts to the latter. The record shows 53 books purchased, 41 by the Marine Biological Laboratory and 12 by the Woods Hole Oceanographic Institution; 18 presented by the authors and 39 from publishers; and Dr. Knower presented to the Library a biography of William B. Wherry by M. Fischer. Completed back sets of serials number 34; as purchases of the Marine Biological Laboratory, 27; of the Woods Hole Oceanographic Institution, 1; by exchange with the "Biological Bulletin," 2; by exchange of duplicate material, 1; and by gift, 3; while purchases of the Marine Biological Laboratory partially complete back sets, 13; exchanges of the "Biological Bulletin," 3; exchanges of duplicate material, 5; and by gift, 3. Reprint additions number 3850; current for 1938, 1559; current for 1939, 632, and of previous dates, 1659. Of these 432 pamphlets came to the Woods Hole Oceanographic Institution. A summary of the current holdings for the Library is therefore 46,136 bound volumes and 112,777 reprints.

VI. THE REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen: I present herewith a report of the fifty-second session of the Marine Biological Laboratory, for the year 1939.

1. *Recovery from Flood Damage.* During the past year the Laboratory has gradually recovered from the extensive damages caused by the hurricane and flood of September, 1938. The task of putting to rights the very great amount of apparatus, of replacing what could not be repaired, of rebuilding drawers and shelving, and of restoring the chemical room to working order, has necessarily been long in completion. But under the competent direction of our permanent staff it is now well along. We may take pride in the fact that during the summer of 1939 no one was seriously inconvenienced by lack of usual laboratory facilities or other services ordinarily provided. For this happy outcome we are indebted particularly to Dr. Pond and to Mr. Larkin who have given unsparingly of their time and skill.

2. *Plans for New Buildings.* The Committee on Policies and Future of the Laboratory, in its report published last year, stressed three main needs: (*a*) additional stack-room space for the Library; (*b*) the replacement of the present wooden buildings with a fire-proof structure of solid construction; and (*c*) increased facilities for the Supply Department. Plans for all three projects have been drawn up. The Library Committee has approved of the preliminary plans for an addition to the Library. This is to be placed at the north end of the Brick Building extending in the same direction, but set back to overlap only the width of the present stack room. By this arrangement the reading room will not be disturbed, while the new stacks will be assured plenty of light and ventilation. The additional space thus provided will undoubtedly be sufficient to house the increment of books, periodicals, and reprints for many years to come, and will be adequate for a large number of readers.

Tentative plans for a building to replace the old wooden buildings have been prepared. They provide increased space for class rooms, larger individual laboratories for instructors and other investigators, a lecture hall, conference rooms, and adequate space for various facilities required for experimental work. The building, which according to the present plans will extend from the site of the Lecture Hall eastward along Center Street, will house about the maximum number of investigators and students who now work in the present old laboratories. It is not the intention to provide for any substantial increase over that maximum.

The Committee on the Supply Department, in collaboration with Mr. Norris Jones, has prepared plans for utilizing more efficiently the buildings and space now occupied. They believe that the character of the present structures is, in general, consistent with the local surroundings and should be preserved. The Stone House will be little changed outwardly, but rebuilt within so as to provide space for the Museum now in preparation by Mr. Gray. A portion of the present Supply Department building will be transformed into laboratories adapted particularly for those who will carry on studies in the natural history of the Woods Hole region. To aid in this work, and to extend the area in which collections can be made, another boat, capable of fair speed, is needed.

In the preparation of all of these plans the Committees have had the whole-hearted coöperation of the architect, Mr. F. V. Bulfinch, who made numerous trips to Woods Hole to study the situation and to consult with the various members.

The Committee on Research and Equipment points out that new experimental procedures are constantly coming into use, and that it is impossible to foresee what may be needed in the future. For that reason they recommend that a special fund be set aside to provide for such contingencies. For the present, a number of highly desirable improvements should be made. Among these are additional de-humidifying rooms, constant temperature rooms, sterilizing and dark rooms, and shop space for investigators. Place for these facilities can be provided in the new building and in certain rooms of the Brick Building.

To finance these various projects, and some others which should be undertaken in the near future, and to add substantially to our endowment, approximately $1,000,000 will be needed. The Committee has sent to the Rockefeller Foundation, for their consideration, a statement of our needs, together with other pertinent information regarding the Laboratory.

3. *Acknowledgments.* The Laboratory is greatly indebted to the Rector of the Church of the Messiah at Woods Hole, Rev. Robert W. Nicholson, who has generously allowed us to use the greenhouse located in the rear of the Rectory. Its constant use during the summer by many investigators emphasizes the fact that we are in urgent need of a greenhouse of our own.

We are also indebted to the National Research Council whose grant made possible the purchase of an X-ray tube much needed in carrying on the work of this rapidly growing department of research.

Dr. Oliver Strong has made a substantial and very welcome contribution by selling to the Laboratory, at a price far below the market

value, his lot on the bathing beach. Funds for securing this property were provided by a generous friend of the Laboratory who wishes to remain anonymous. By this timely action we are now assured permanent possession of an adequate beach.

5. *Election of Trustees and Officers.* At the meeting of the Trustees in 1939, Prof. G. N. Calkins was elected Trustee Emeritus. The new Trustees elected at that meeting were Dr. J. H. Northrop and Dr. W. R. Taylor. As Secretary of the Trustees, to take the place of Prof. Calkins, who ably served in this capacity for many years, Dr. H. B. Goodrich was chosen.

6. *Memorials.* The Memorials of deceased Trustees, read at the Corporation meetings held in 1938 and 1939, are printed in this report.

7. *Scientific Record of Former Students.* A brief statement of the record of former students in our courses was presented at the 1939 meeting of the Trustees. The analysis of the data was subsequently completed and is included in this report. The conclusion may fairly be drawn that the courses of instruction are fulfilling their purpose in a most gratifying manner.

There are appended as parts of this report:

1. Memorials of deceased Trustees.
2. A report on the scientific record of students attending during the years 1918–1931.
3. The Staff, 1939.
4. Investigators and Students, 1939.
5. A tabular view of attendance, 1935–1939.
6. Subscribing and Coöperating Institutions, 1939.
7. Evening Lectures, 1939.
8. Shorter Scientific Papers, 1939.
9. The General Scientific Meeting, 1939.
10. Members of the Corporation, 1939.

Respectfully submitted,

CHARLES PACKARD,
Associate Director.

I. MEMORIALS OF DECEASED TRUSTEES

I. Memorial to Dr. E. P. Lyon

By W. E. Garrey

Elias Potter Lyon died on May 4, 1937 in his seventieth year. For forty years he was a staunch friend and supporter of the Marine Bio-

logical Laboratory. As a graduate student at the University of Chicago he conducted his early investigations in this laboratory. In 1898 he was appointed one of the teaching staff which organized the first course in physiology given at Woods Hole in 1899. As an active investigator he published many reports of work done here; these dealt with the animal tropisms, other phases of animal behavior and reactions to environment. He also contributed largely to studies on the early stages of embryonic development, particularly the rhythmic oxidation and chemical changes incident to egg cleavages. He possessed a remarkable ingenuity in devising simple methods and decisive experiments, probably the outgrowth of early training and teaching of physics. His colleagues well remember the hum and whir of centrifuges which followed his introduction of that method of studying organization in marine eggs.

Lyon taught physics at the Harvard School in Chicago, biology at Bradley Polytechnic Institute (Peoria, Illinois), and physiology successively at Rush Medical College, University of Chicago, St. Louis University Medical School and University of Minnesota.

As a teacher of physiology he impressed upon his students the importance of fundamental biology and he induced and aided many of them to study at the Marine Biological Laboratory. As Dean of the Medical Schools at St. Louis and Minnesota he exhibited a fine administrative sense and capability which was often demonstrated while he was a Trustee of this Laboratory from 1922 to 1930—a tenure he resigned only when his heavy administrative duties limited his Woods Hole contacts.

Interested ever in their welfare, Lyon was the true and sympathetic friend of youth; to his maturer intimates he was loved for his innate tolerance, kindliness and generosity—for his absolute lack of selfishness. These attributes coupled with his high scientific and scholarly attainments and his whole-hearted devotion to the Marine Biological Laboratory are the marks of his distinction among us. Therefore, be it

Resolved: That the Corporation of the Marine Biological Laboratory record in its minutes an acknowledgment of its indebtedness to Elias Potter Lyon for his many years of service to, and association with the Laboratory, and its sense of the deep loss sustained by his death. Be it further

Resolved: That a copy of these sentiments and resolutions be transmitted to his widow, Mrs. Nelle Eastman Lyon.

August 9, 1938

II. Minutes on the Death of Milton J. Greenman

By C. R. Stockard

The Corporation of the Marine Biological Laboratory record a sense of deep loss at the death of Milton J. Greenman which occurred on April 7th, 1937.

Doctor Greenman became a Trustee of this Laboratory in 1908 and served actively for twenty-eight years until he was made Trustee Emeritus in 1936. Throughout the time of his Trusteeship he was engaged as Director of the Wistar Institute and the relationship between that Institute and this Laboratory was most intimate and cordial. Through Doctor Greenman's good offices the Wistar Institute subscribed for five research rooms at this laboratory for a number of years during a period when its economic problems were very difficult. The generous arrangement provided that the occupants of the Wistar Institute rooms were to be appointed by the Director of this Laboratory and few supporting institutions have been so liberal in this regard.

At the Wistar Institute Doctor Greenman not only developed a scientific staff in a place which had previously been a museum, but he soon became convinced that the Institute could render great service in aiding the anatomical and biological publications of the country. Thirty years ago, in 1908, five morphological periodicals of national importance were brought together under the publishing management of the Institute. Before this time these journals had depended upon the efforts of private individuals and in 1903 one of the publications, the Journal of Morphology, of which an earlier Director of this laboratory had been co-founder, had ceased publication. Through the Wistar Institute, Doctor Greenman renewed publication of the Journal of Morphology. The ownership of the journals was for some time in the hands of the editorial boards but all are now the property of the Wistar Institute. Through the publication of these journals Doctor Greenman rendered broad service to the workers in this Laboratory and those in all biological laboratories of the country. Therefore, be it

Resolved: That Doctor Greenman's efforts in these several directions and his long interest and constant attendance at the annual meetings of this Laboratory are deeply appreciated by the Corporation and Trustees, and be it

Resolved: That a copy of these sentiments and resolutions be recorded in the Minutes of the Corporation, and that a copy be sent to the family of Dr. Greenman.

III. Memorial to Professor William Morton Wheeler

By G. H. Parker

William Morton Wheeler died suddenly in Cambridge, Massachusetts, April nineteenth, 1937, in his seventy-third year.

Professor Wheeler became a member of the Corporation of the Marine Biological Laboratory in 1890 and, with the exception of a few years, he remained so until his death. He worked in the Laboratory as early as 1891, only three years after its founding, and participated in the Nature Study Course given here in 1900. He was elected a Trustee of the Laboratory in 1919 and served in that capacity till 1935 when he was made a Trustee Emeritus.

Professor Wheeler's distinguished career as a naturalist began in his native town of Milwaukee where his interest in insects was fostered by Dr. and Mrs. G. W. Peckham. His acquaintance with the life of the sea was first made in this laboratory under Dr. Whitman and was further strengthened by work at the Zoölogical Station at Naples. In his early days, marine problems claimed a fair share of his attention, but as time went on he became more and more occupied with the study of insects and especially of ants. Of the three hundred and more contributions from his pen, the majority of them have to do with these animals. He was the recipient of many honors and distinctions, and in his death the world of science and the Marine Biological Laboratory in particular loses an eminent teacher and a renowned investigator.

Whereas: Professor Wheeler, whose high distinction in biological research is so widely known, was a Trustee of the Corporation of the Marine Biological Laboratory for some eighteen years, therefore, be it

Resolved: That the Trustees and Corporation of the Marine Biological Laboratory recognize with great sorrow that in the death of Professor Wheeler science has lost an unusual scholar and this Corporation a distinguished Trustee and a loyal advocate. And be it further

Resolved: That this resolution be adopted and recorded in the minutes of the Corporation and that a copy of it be sent to the family of Professor Wheeler.

IV. Memorial to Dr. H. H. Donaldson

By E. G. Conklin

In the death of Henry H. Donaldson on January 23, 1938, the Marine Biological Laboratory lost one of the original members of its Corporation, and a Trustee for more than 25 years. Dr. Donaldson became a member of the Corporation in 1888 and continued an annual

membership until his death. In the report of the Treasurer for 1888 he is listed as one of the original contributors to the support of the Laboratory. He was elected a Trustee in 1912 and re-elected in 1916, 1920, 1924; in 1929 he was elected Trustee Emeritus. With the exception of the sessions of 1920 and 1928, he occupied a research room at the Laboratory every summer after 1912. He served on various committees of the Trustees and his sound judgment and wise counsel were always available and of great value. Therefore, be it

Resolved: That the Corporation and Trustees hereby record their sorrow in the loss of a loyal and helpful member of our organization, a man of genuine scientific temper and of high ideals, and a personal friend of whom we have only happy and grateful memories. And be it further

Resolved: That these sentiments and resolutions be recorded in the minutes of the Corporation and that a copy be sent to Mrs. Donaldson.

IV. Memorial to Dr. E. B. Wilson

By Gary N. Calkins and T. H. Morgan

(Read by Dr. Calkins)

Edmund Beecher Wilson was born in Geneva, Illinois, in 1856, a son of Isaac G. Wilson and Caroline Clark Wilson. He attended Antioch College, the old University of Chicago, and the Sheffield Scientific School of Yale where he obtained the degree of Bachelor of Science in 1878; and three years later the degree of Ph.D. at Johns Hopkins University. He then studied in Cambridge, England, in Leipzig, and in Naples. During this period he made deep and lasting friendships with many of the leaders of European biology—Boveri, Butschli, Dohrn, Driesch, R. Hertwig and many others.

On returning to America, Wilson lectured for a year at Williams College and at the Institute of Technology. From 1885 to 1891 he was Professor of Biology at Bryn Mawr College. He was then called to Columbia University as Adjunct-Professor of Biology. Later he became Professor of Invertebrate Zoölogy, and then da Costa Professor of Zoölogy, holding the latter position until he was retired in 1928 with the title Professor Emeritus of Zoölogy in Residence in Columbia University. He died in New York City on March 3rd last.

Wilson's connection with the Marine Biological Laboratory began almost at its start in 1888, as he became a Trustee in 1889 and remained on the Board for the remainder of his life. Keenly interested in the welfare of the Laboratory, he took an active part in all the meetings of the Board.

Active and vigorous in youth and middle age, he was ready for athletic activity of all kinds, but bicycling, golf and sailing were the sports that he apparently liked best. His sensitive, artistic nature found ample expression in music and for many years not only did he patronize the musical centers of New York, but he was a devoted and excellent cello player, a member of a well-known New York string quartet, and a prominent member of the musical circles of that city.

He was a member of all of the leading learned societies of this country and Europe, and the recipient of innumerable honors here and abroad. But, significant as these are, he will be lovingly remembered by his many friends for the distinction of his mind and personality. His scientific keenness, judgment, and breadth of knowledge, were shown by the perfection of his lectures and papers.

He was the recognized leader in cytological research. His book on "The Cell in Development and Heredity" remains and will long remain a classic in this field. The American school of cellular research was, in large part, the outcome of his influence. His many devoted students and friends will remember him as their ideal of a scientific worker and charming companion.

V. Memorial to Dr. Charles R. Stockard

By Dayton J. Edwards

The Corporation of the Marine Biological Laboratory records with profound regret the death on April 7th, 1939 of Charles Rupert Stockard, a member of this scientific body since 1908 and an active participant in its affairs through a consecutive service of nineteen years on its board of Trustees.

Professor Stockard was born in Washington County, Mississippi, where his father was a practitioner of medicine. At an early age he was thus brought in touch with many of the every-day aspects of human biology and the sociological problems of the community. This heritage and these early experiences left imprints of deep significance in his life which in later years he frequently commented on; sometimes blending them into present-day situations and at other times contrasting them in effective and meaningful ways.

A preliminary college education in the Mississippi Agricultural and Mechanical College was completed in 1899 and soon thereafter he entered graduate work at Columbia University. In 1906 he became associated with the teaching staff of Cornell University Medical College. He was made Assistant Professor of Embryology in 1909 and two years later he was appointed Professor of Anatomy and Director of that De-

partment, a post he held for the remaining years of his life. Coincident with his academic assignments he maintained a vital interest in the Marine Biological Laboratory. This Institution held for him a peculiarly deep-seated significance and he was wont to recall his early associations here; also, the friendships with his old teachers, with his contemporaries, and with the group of younger biologists.

A bibliography covering a wide range of topics in the fields of cytology, embryology, genetics, endocrinology, medicine and education conveys at once an idea of Professor Stockard's versatility and symbolizes in a concrete way something of the genius, the originality, and the scholarliness of his mind. Approximately thirty-five of his earlier papers deal with problems on regeneration and the artificial production of structural anomalies in lower forms. His commanding knowledge of these problems was reflected prominently in much of his later work and teachings.

His engaging personality makes his passing a far greater loss than is indicated by his scientific achievement. A free and entertaining conversationalist, he was able to turn to the lighter sides of life with facility and enjoyment, and to witticisms of the most humorous nature. Endowed with a mind ready to challenge any height, and prompt to champion any cause he believed to be right, he possessed also a keen sense of scientific values, an incisive way of thinking and an unadorned form of expression.

In Charles Rupert Stockard, death has taken from this Corporation of the Marine Biological Laboratory one of the staunchest champions, one of its must faithful servants and helpful mentors.

Be it therefore Resolved: That the Members of the Marine Biological Laboratory hereby express their deep sense of the great loss which they have suffered through the sudden and untimely taking off of their fellow.

VI. Memorial to C. R. Crane

By Gary Calkins and T. H. Morgan

(Read by Dr. Morgan)

Charles R. Crane's beginning interest in this Laboratory can be placed approximately in 1901 when he was elected a member of the Corporation and a Trustee. But even before that date he had personal contacts with the work of the Laboratory which continued throughout his life. He became President of the Corporation in 1903, succeeding Professor H. F. Osborn, who resigned at that time. As soon as he became a Trustee he identified himself with the ideals and interests of the Laboratory.

His benefactions began in 1901, through personal gifts. When the contributions from the Carnegie Institution that extended from 1903 to 1905 came to an end, the Laboratory lived from hand to mouth, as it were, depending on its friends to cover its growing deficits. From 1910 to 1924 Mr. Crane, by most generous gifts, carried the burden of operating deficits almost alone. In addition he presented the first permanent building in 1913. In 1924 and 1925, in conjunction with the Rockefeller Foundation, Mr. John D. Rockefeller, Jr., and the Carnegie Corporation, Mr. Crane contributed largely to the erection and equipment of the second permanent building and to a sufficient endowment for future operations.

Quoting Dr. Lillie:

"The history of the institution would have been very different indeed if it had not been for Mr. Crane. His support gradually developed confidence in the soundness of the institution, even though managed by professors, and was a major factor in securing large gifts from the Rockefeller Foundation, Mr. John D. Rockefeller, Jr., the General Education Board and the Carnegie Corporation."

At the dedication of the Crane Laboratory in 1914 Mr. Crane said:

"I think we have come here particularly to celebrate the wonderful spirit that is back of the Woods Hole Biological Laboratory. It is very difficult to define that spirit, but I think we all know something of it and something is also known all through the scientific world. Without that spirit no amount of bricks and mortar and organization would be of any great service, but with that spirit the laboratory has been able to accomplish a very great deal with very simple means." (Director's Report for 1914, BIOLOGICAL BULLETIN, Vol. 28, No. 6, 1914, p. 345.)

Dr. Lillie commented recently: "This statement, characterized by brevity, appreciation of others, understanding of aims, and quiet humor gives admirably the spirit that animated all his acts for the Laboratory."

Let me quote, again, from Mr. Crane's address in 1925 on the occasion of the dedication of the second permanent laboratory:

"Even though not personally associated with the vital processes of the Laboratory, it has been the greatest possible privilege to play the part of a simple spectator in watching the growth of the wonderful spirit of coöperation in the work of biological research.

"Some years ago the then business manager of Rockefeller Institute for Medical Research invited me to spend the evening with him and try to help him understand the nature and conditions of the spirit of the Marine Biological Laboratory. 'For,' said he, 'We all recognize that the spirit is there. It is the rarest thing that we know of, and we have many discussions as to its nature and the conditions under which it has come forth.' He then asked me if I had any theory about it. I answered that the essential thing, as it

seemed to me, was that it was the purest expression of the highest form of democracy—a form of Soviet directed by the highest rather than the lowest motives. . . .

"We all know that this spirit which we are so much concerned about has long been domesticated in the old buildings across the street and among the older biologists. Although the street is a very narrow one, the mission of inviting the spirit of the laboratory into the new and more modern buildings and giving it a longer lease of its great power is mainly up to the younger biologists now coming along. Much power to them!" (From *Science,* Vol. 62, 1925, 271–272.)

When, in 1925, Mr. Crane resigned as President of the Trustees, he wrote:

"Twenty-two years have now elapsed since I became President of the Marine Biological Laboratory. I have enjoyed with you watching the growth of the Laboratory during that period. With the strong interest and support that is now assured, I feel that my own work has been completed and I hereby tender you my resignation which I ask you to accept. . . .

"The future progress and prosperity of the Laboratory will always be a matter of great interest to me, quite as much as if I continued to be your President."

Other gifts he has made from time to time, most of them of great value to the Laboratory, but the gift that the members of the Laboratory will cherish as being the greatest was his appreciation and understanding of the work that has been carried on in the Marine Biological Laboratory.

Mr. Crane died February 14, 1939.

VII. Memorial to Dr. J. P. McMurrich

By Frank R. Lillie

James Playfair McMurrich, Emeritus Professor of Anatomy in the University of Toronto, died February 9, 1939 in his seventy-ninth year. He was born and educated in Toronto and received his early training in zoölogy there; he obtained his Ph.D. degree in zoölogy at Johns Hopkins University in 1885. He received the honorary degree of LL.D. from the University of Michigan in 1912, from the University of Cincinnati in 1923 and from the University of Toronto in 1931. He held numerous offices in scientific societies, including the presidency of the American Association for the Advancement of Science in 1922.

His entire life was one of great scientific activity in research, teaching, and administration. He was primarily a zoölogist; although the greater part of his life was spent as Professor of Anatomy in a medical school, he never took a medical degree. He published 107 scientific papers from 1882 to 1932, was the author of two text books well-known in their time, "Invertebrate Morphology" (1894) and "Development

of the Human Body" (1903), and edited two treatises on human anatomy. His research interests were wide, ranging from coelenterates, mollusks, crustaceans and ascidians to various vertebrate groups; and in the last years of his life, the history of anatomy.

At the Marine Biological Laboratory we remember him best in his invertebrate days. He came to the Marine Biological Laboratory first in 1889 while serving as an associate of Professor Whitman in Clark University. In that year, and for two succeeding years, he was instructor in the course in Invertebrate Zoölogy. He delivered evening lectures on "The Phylogeny of the Actinozoa" (1889), "The Gastrea Theory and Its Successors" (1890) and on "The Significance of the Blastopore" (1891). He was a member of the Corporation from 1890 to the time of his death, and from 1892 to 1901 he was a member of the Board of Trustees. His withdrawal from active participation in the affairs of the Marine Biological Laboratory was a natural consequence of the change in direction of his scientific work to anatomy in relation to medicine.

It is many years since McMurrich was actively concerned in our affairs, though his interest was lifelong; but there are still some of us who remember him at Woods Hole as a quiet, courteous gentleman and scholar, loyal to the interests of the Laboratory, a good friend, fond of life and sports, and withal of exceptionally fine character and quality.

VIII. Memorial to Professor Edwin Linton

By G. H. Parker

Professor Edwin Linton, a member of the Corporation of the Marine Biological Laboratory since 1898, died in Philadelphia June 4, 1939, in his eighty-fifth year. He came to Woods Hole first in 1882 and worked in the Laboratory of the United States Fish Commission temporarily located on the Buoy Wharf at Little Harbor. At the time of his death he was the last survivor of that original band of investigators who first brought biology to this immediate shore. From these early times till his death Dr. Linton was an unremitting student of the parasites of fishes, a subject to which he made many valuable and original contributions. Although his researches were carried out in the Laboratory of the Bureau of Fisheries he always took a keen and active interest in the affairs of the Marine Biological Laboratory. He became a corporate member of this institution in the year when, by invitation, most of the workers at the Fisheries Laboratory joined this Corporation, whose annual meetings he regularly attended.

He was a familiar figure to all who came to the Woods Hole Laboratories. He took a lively interest not only in the general scientific

activities of the community, but also in its play. He was a skilled actor, the first President of the reorganized M. B. L. Club, and an enthusiastic member of the Choral Club. He and his wife established at Washington and Jefferson College the "Edwin S. Linton Memorial Endowment" in memory of their son who gave his life in France during the World War. The income from this endowment is paid to the Marine Biological Laboratory and has been the means of enabling not a few students to work here. This and other generous acts associated with Dr. Linton's name will long be remembered. Dr. Linton's sense of social obligation led him always to be a willing participant in any movement for the general good. In fact, it was through a step to protect others that he met with the accident that cost him his life. We mourn his loss as that of an earnest, scientific worker and of a generous and loyal associate. Therefore, be it

Resolved: That the Corporation of the Marine Biological Laboratory records in its minutes its sense of the great loss it has sustained in the death of Edwin Linton and be it further

Resolved: That a copy of these sentiments and resolutions be transmitted to Dr. Linton's family.

IX. Memorial to Dr. C. B. Bridges

By A. H. Sturtevant

Dr. Calvin Blackman Bridges died on December 27, 1938, just a few days before reaching the age of 50 years.

Dr. Bridges first came to the Marine Biological Laboratory in 1912, while he was still an undergraduate. For the next twenty years he regularly spent the summers at Woods Hole, where much of his work on the genetics and cytology of *Drosophila* was done.

Dr. Bridges had an unrivalled familiarity with this material, and one of his outstanding characteristics was his readiness to help other workers by supplying laboriously prepared material and by giving freely of his time in an advisory capacity. His scientific work was a conspicuous example of the unselfish coöperation that is one of the ideals of the Marine Biological Laboratory.

X. Memorial to W. O. Luscombe

By Caswell Grave and F. M. MacNaught

(Read by Dr. Grave)

For forty-three years Walter O. Luscombe has been an interested member of the Corporation of the Marine Biological Laboratory. He

joined in 1896 at a time when the Laboratory needed friends and encouragement; before its future had been assured by a large and devoted membership of biologists and by contributions of substantial buildings and endowment.

Mr. Luscombe sensed the worth of the struggling institution that was seeking to make its home in his community, and his request for membership in the Corporation is to be viewed as the action of a responsible citizen giving public expression to the interest and good will of the whole community.

He died in July in his 88th year.

We shall miss him from our Annual Meetings which he regularly attended and we find satisfaction in recording our appreciation of his kindly interest and good will.

2. THE SCIENTIFIC RECORD OF STUDENTS IN COURSES AT THE MARINE BIOLOGICAL LABORATORY

In the report of the Committee on the Policies and Future of the Marine Biological Laboratory the statement is made that "We have stood by the principle that it is the business of the Laboratory to help to produce investigators as well as investigation; and we believe that it can be shown that our courses of instruction have contributed in an important way to this purpose, and, moreover, that they have been an important factor in the improvement of biological instruction and research throughout the country." The record of the students who have attended these courses in past years fully justifies this belief. A very satisfactory proportion has continued in productive research in colleges and universities.

The first analysis of student records was made by Dr. G. A. Drew in 1923. His results, which cover the years 1908–1917 inclusive, are shown in the accompanying table. Those students whose names appeared in the current edition of American Men of Science were regarded as eminently successful teachers and investigators.

	Total	In A. M. S.
Invertebrate Zoölogy	350	12 per cent
Embryology	197	24 " "
Physiology	93	20 " "
Botany	111	26 " "
	751	18 " "

Out of a total of 751 registered during these years, 136 or 18 per cent

are thus cited. This record was considered excellent; but the figures for subsequent years are still more satisfactory.

The present analysis begins with 1918 and extends through 1931. A total of 1776 students were enrolled, of whom 56 were residents of foreign countries. Since the latter are not eligible for citation in American Men of Science, their records are not included here.

The second table shows the total number of men and women in each course, the number of these mentioned in American Men of Science (1938 edition) and the percentage of successful students, judged by this criterion. In the column labelled All Students, it is seen that out of 1720, 509 men and women, that is, 30 per cent have been included. The record of the men is uniformly much better than that of the women (50 per cent against 14 per cent). The low score of the latter is not a reflection of their ability. A large proportion marry and cannot continue in scientific work. Furthermore, there are not many opportunities for women to pursue independent research. In the column labelled "Corr." the figures are corrected to account for students who have taken more than one course. Except in the case of Protozoölogy, these corrections are not significant.

In the remaining sections of the table the students have been classified in three categories; (1) Undergraduates; that is, those who have not begun graduate work; (2) Graduate students; and (3) Instructors, including all teachers from instructors to those of professorial rank. The chief point of interest here is the fact that in each category, the proportion of men whose names appear in American Men of Science is about the same in all of the courses. Thus, among the undergraduates this is about 36 per cent for each course; among the graduates it is 67 per cent. The only exception in the latter group is in Botany. But in view of the small number of men (6) the low score is not significant. The same comment applies to the percentages of instructors.

The record for the women students in the different classes in each of the three categories is decidedly lower than that of the men. Taking into consideration the fact already mentioned that many have no opportunity for continuing their scientific work, the proportion cited in American Men of Science is good.

In general, therefore, it appears that of all students attending courses at Woods Hole, about one in three are mentioned in American Men of Science. Among the men alone, fully half attained this distinction, a remarkably good record. While it would be wrong to assume that such students have been successful because they attended the summer courses, yet it is true beyond all doubt that they received at Woods Hole a stimulus which was an important factor in directing their scientific career.

Record of Students in the M.B.L. courses during 1918–1931 inclusive

		All Students			Corr.	Under-graduates			Graduates			Instructors		
		Total	AMS	Per Cent		Total	AMS	Per Cent	Total	AMS	Per Cent	Total	AMS	Per Cent
Invert. Zoology	M	308	144	47.0	47.0	213	78	36.9	61	41	67.2	34	25	73.5
	W	441	46	10.5	10.6	378	37	10.0	45	6	13.3	18	33	16.7
	T	749	190	25.4	25.4	591	115	19.5	106	47	43.3	52	28	53.8
Embry-ology	M	174	80	46.0	49.0	113	39	35.1	27	18	66.7	34	23	67.7
	W	197	35	17.8	17.7	143	19	13.3	31	7	22.6	23	9	31.3
	T	371	115	31.0	33.6	256	58	22.7	58	25	43.1	57	32	56.1
Physi-ology	M	126	70	55.6	59.6	56	20	35.7	43	30	70.0	27	20	74.1
	W	107	25	23.4	23.5	60	10	16.8	26	8	30.8	21	7	33.3
	T	233	95	40.8	42.2	116	30	25.9	69	38	55.1	48	27	56.3
Proto-zool.	M	100	57	57.0	64.7	37	13	35.1	27	18	66.7	36	26	72.2
	W	90	15	16.7	22.2	48	5	10.4	23	6	26.1	19	4	21.1
	T	190	72	38.0	45.9	85	18	21.2	50	24	48.0	55	30	54.6
Botany	M	50	21	42.0	44.7	34	12	35.3	6	3	50.0	10	6	60.0
	W	127	16	12.6	12.7	76	4	5.3	19	4	21.1	32	8	25.0
	T	177	37	20.9	21.4	110	16	15.5	25	7	20.0	42	14	33.0
Totals	M	758	372	49.1	51.3	453	162	35.8	164	110	67.1	141	100	70.9
	W	962	137	14.2	14.3	705	75	10.6	144	31	21.5	113	31	27.4
	T	1720	509	29.6	30.7	1158	237	20.5	308	141	45.8	254	131	56.0

Note: 56 foreign students have been omitted since they are not eligible in AMS.

3. THE STAFF, 1939

Charles Packard, Associate Director, Assistant Professor of Zoölogy, Institute of Cancer Research, Columbia University.

ZOÖLOGY

I. Investigation

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
E. G. Conklin, Professor of Zoölogy, Princeton University.
Caswell Grave, Professor of Zoölogy, Washington University.
H. S. Jennings, Professor of Zoölogy, University of California.
Frank R. Lillie, Professor of Embryology, Emeritus, The University of Chicago.

C. E. McClung, Professor of Zoölogy, University of Pennsylvania.
S. O. Mast, Professor of Zoölogy, Johns Hopkins University.
T. H. Morgan, Director of the Biological Laboratory, California Institute of Technology.
G. H. Parker, Professor of Zoölogy, Emeritus, Harvard University.
Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. Instruction

T. H. Bissonnette, Professor of Biology, Trinity College.
P. S. Crowell, Jr., Instructor in Zoölogy, Miami University.
F. R. Kille, Assistant Professor of Biology, Swarthmore College.
A. M. Lucas, Associate Professor of Zoölogy, Iowa State College.
S. A. Matthews, Assistant Professor of Biology, Williams College.
J. S. Rankin, Jr., Instructor in Biology, Amherst College.
A. J. Waterman, Assistant Professor of Biology, Williams College.

Junior Instructors

W. E. Martin, Assistant Professor of Zoölogy, DePauw University.
N. T. Mattox, Instructor in Zoölogy, Miami University.

PROTOZOÖLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Gary N. Calkins, Professor of Protozoölogy, Columbia University.
Virginia Dewey, Research Assistant, Brown University.
G. W. Kidder, Assistant Professor of Biology, Brown University.

EMBRYOLOGY

I. Investigation

(*See Zoölogy*)

II. Instruction

Hubert B. Goodrich, Professor of Biology, Wesleyan University.
W. W. Ballard, Assistant Professor of Biology and Anatomy, Dartmouth College.
Donald P. Costello, Assistant Professor of Zoölogy, University of North Carolina.
Viktor Hamburger, Assistant Professor of Zoölogy, Washington University.
Oscar Schotté, Associate Professor of Biology, Amherst College.

PHYSIOLOGY

I. Investigation

William R. Amberson, Professor of Physiology, University of Maryland, School of Medicine.
Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.
Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.
M. H. Jacobs, Professor of General Physiology, University of Pennsylvania.
Ralph S. Lillie, Professor of General Physiology, The University of Chicago.
Albert P. Mathews, Professor of Biochemistry, University of Cincinnati.

II. Instruction

Teaching Staff

Laurence Irving, Professor of Biology, Swarthmore College.
Robert Chambers, Professor of Biology, New York University.
Kenneth C. Fisher, Assistant Professor of Experimental Biology, University of Toronto.
Rudolf Höber, Visiting Professor of Physiology, University of Pennsylvania.
C. Ladd Prosser, Assistant Professor of Physiology, Clark University.
James A. Shannon, Assistant Professor of Physiology, New York University Medical College.
F. J. M. Sichel, Instructor in Physiology, University of Vermont, College of Medicine.

BOTANY

I. Investigation

S. C. Brooks, Professor of Zoölogy, University of California.
B. M. Duggar, Professor of Physiological and Economic Botany, University of Wisconsin.
D. R. Goddard, Assistant Professor of Botany, University of Rochester.
E. W. Sinnott, Professor of Botany, Barnard College.

II. Instruction

Wm. Randolph Taylor, Professor of Botany, University of Michigan.
G. W. Prescott, Associate Professor of Biology, Albion College.
B. F. D. Runk, Research Fellow, University of Virginia.

GENERAL OFFICE

F. M. MacNaught, Business Manager.
Polly L. Crowell, Assistant.
Edith Billings, Secretary.

RESEARCH SERVICE AND GENERAL MAINTENANCE

Samuel E. Pond, Technical Mgr.
G. Failla, X-ray Physicist.
Elbert P. Little, X-ray Technician.
J. D. Graham, Glassblower.
T. E. Larkin, Superintendent.
Lester F. Boss, Technician.
W. C. Hemenway, Carpenter.
J. T. Simonton, Assistant.

LIBRARY

Priscilla B. Montgomery (Mrs. Thomas H. Montgomery, Jr.), Librarian.
Deborah Lawrence, Secretary.
Mary A. Rohan, S. Mabell Thombs, Assistants.

SUPPLY DEPARTMENT

James McInnis, Manager.
Milton B. Gray, Collector.
A. M. Hilton, Collector.
A. W. Leathers, Shipping Dept.
Geoffrey Lehy, Collector.
Walter Kahler, Collector.
F. N. Whitman, Collector.
Ruth S. Crowell, Secretary.
Grace Harman, Secretary.

4. INVESTIGATORS AND STUDENTS

Independent Investigators, 1939

Abell, Richard G., Instructor in Anatomy, University of Pennsylvania, School of Medicine.
Abramowitz, A. A., Research Assistant, Harvard University.
Addison, William H. F., Professor of Normal Histology and Embryology, University of Pennsylvania, School of Medicine.
Allee, W. C., Professor of Zoölogy, The University of Chicago.
Amberson, William R., Professor of Physiology, University of Maryland, School of Medicine.
Andersch, Marie, Assistant Professor, Woman's Medical College of Pennsylvania.
Anderson, Rubert S., Biophysicist, Memorial Hospital, New York City.
Angell, Nancy, Bryn Mawr College.
Angerer, Clifford A., Instructor in Zoölogy, University of Pennsylvania.
Bailey, Kenneth, Rockefeller Fellow, Harvard University Medical School.
Ball, Eric G., Associate in Physiological Chemistry, Johns Hopkins University Medical School.
Ballard, William W., Assistant Professor, Dartmouth College.
Barth, Lester G., Assistant Professor of Zoölogy, Columbia University.
Beams, H. W., Associate Professor of Zoölogy, State University of Iowa.
Beck, Lyle V., Instructor in Physiology, Hahnemann Medical School.
Bissonnette, T. Hume, Professor of Biology, Trinity College.
Boche, Robert D., Research Assistant, Carnegie Institution of Washington.
Boell, E. J., Instructor in Zoölogy, Yale University.
Botsford, E. Frances, Assistant Professor of Zoölogy, Connecticut College.
Brill, Edmund R., Graduate Student in Biology, Harvard University.
Bronfenbrenner, J. J., Professor of Bacteriology, Washington University, School of Medicine.
Brown, Dugald, Assistant Professor, New York University, College of Medicine.
Brown, Frank A., Jr., Assistant Professor of Zoölogy, Northwestern University.
Brown, Morden G., Research Associate, Washington University.

Buck, John B., Research Assistant, Carnegie Institution of Washington.
Cable, Raymond M., Assistant Professor of Parasitology, Purdue University.
Calkins, Gary N., Professor Emeritus of Protozoölogy, Columbia University.
Carpenter, Russell L., Professor of Zoölogy, Tufts College.
Chambers, Robert, Research Professor of Biology, Washington Square College, New York University.
Chase, Aurin M., Research Associate, Princeton University.
Cheney, Ralph H., Chairman, Biology Department, Long Island University.
Claff, C. Lloyd, Research Associate in Biology, Brown University.
Clark, Eleanor L., University of Pennsylvania, School of Medicine.
Clark, Eliot R., Professor of Anatomy, University of Pennsylvania, School of Medicine.
Clement, Anthony C., Assistant Professor of Biology, College of Charleston.
Clowes, G. H. A., Director of Research, The Lilly Research Laboratories.
Clutton, R. F., Student, Columbia University Medical Centre.
Cole, Kenneth S., Associate Professor of Physiology, Columbia University.
Colwin, Arthur L., Instructor, Washington Square College, New York University.
Copeland, Eugene, Assistant in Biology, Harvard University.
Copeland, Manton, Professor of Biology, Bowdoin College.
Cornman, Ivor, Teaching Fellow in Biology, Washington Square College, New York University.
Costello, Donald P., Assistant Professor of Zoölogy, University of North Carolina.
Crowell, Prince S., Jr., Instructor in Zoölogy, Miami University.
Curtis, W. C., Professor of Zoölogy, University of Missouri.
DeMarinis, Frank, Graduate Assistant, Western Reserve University.
Denstedt, O. F., Lecturer, McGill University.
Dieter, Clarence D., Professor of Biology, Washington and Jefferson College.
DuBois, Eugene F., Professor of Medicine, Cornell University Medical College.
Duggar, B. M., Professor of Plant Physiology, University of Wisconsin.
Duryee, William R., Research Associate, Washington Square College, New York University.
Dustin, Pierre, C. R. B. Graduate Fellow, Belgian American Educational Foundation.
Elftman, Herbert, Assistant Professor of Zoölogy, Columbia University.
Evans, Titus C., Associate in Radiology, State University of Iowa.
Failla, G., Physicist, Memorial Hospital, New York City.
Ferry, John D., Junior Fellow of the Society of Fellows, Harvard University.
Figge, Frank H. J., Associate Professor of Anatomy, University of Maryland, School of Medicine.
Finn, James B., Jr., Associate Professor of Biology, Mount Mercy College.
Fisher, Kenneth C., Assistant Professor of Experimental Biology, University of Toronto.
Fowler, Coleen, Assistant Professor, Johns Hopkins University.
Frisch, John A., Professor of Biology, Canisius College.
Fry, Henry J., Visiting Investigator, Cornell University Medical College.
Furth, Jacob, Associate Professor of Pathology, Cornell University Medical College.
Garrey, W. E., Professor of Physiology, Vanderbilt University, School of Medicine.
Geldard, Frank A., Professor of Psychology, University of Virginia.
Goddard, David R., Assistant Professor of Botany, University of Rochester.
Goodrich, H. B., Professor of Biology, Wesleyan University.
Grave, Caswell, Professor of Zoölogy, Washington University.
Guttman, Rita, Tutor, Brooklyn College.

Hadley, Charles E., Associate Professor of Biology, State Teachers' College, Montclair, N. J.
Haff, Richard M., Resident Doctor, Cornell University.
Hamburger, Viktor, Assistant Professor, Washington University.
Hartman, Frank A., Professor of Physiology, Ohio State University.
Harvey, E. Newton, Professor of Physiology, Princeton University.
Harvey, Ethel Browne, Investigator, Princeton University.
Haywood, Charlotte, Associate Professor of Physiology, Mount Holyoke College.
Heilbrunn, L. V., Associate Professor of Zoölogy, University of Pennsylvania.
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Henshaw, Paul S., Research Fellow, National Cancer Institute.
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Höber, Rudolf, Visiting Professor of Physiology, University of Pennsylvania.
Hobson, Lawrence B., Graduate Assistant, University of Cincinnati.
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Howe, H. E., Editor, Industrial and Engineering Chemistry, Washington, D. C.
Hunninen, Arne V., Professor, Oklahoma City University.
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Jacobs, Merkel H., Professor of General Physiology, University of Pennsylvania.
Jenkins, George B., Professor of Anatomy, George Washington University.
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THERMAN, OLOF G., Research Fellow, Harvard University.
TOWNSEND, GRACE, Professor of Biology, Great Falls Normal College.
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VANHEUVERSWYN, JEAN D., Graduate Fellow, Belgian American Educational Foundation.
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WATERMAN, A. J., Assistant Professor of Biology, Williams College.
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WEISSENBERG, RICHARD, Member, Wistar Institute of Anatomy and Biology.

Wenrich, D. H., Professor of Zoölogy, University of Pennsylvania.
White, Thomas N., Jr., Assistant Biophysicist, National Cancer Institute.
Wichterman, Ralph, Instructor, Temple University.
Wickenden, James W., Head of the Biology Department, Deerfield Academy.
Wilhelmi, Raymond, Teaching Fellow, New York University.
Wintrobe, Maxwell M., Associate in Medicine, Johns Hopkins University.
Wolf, E. Alfred, Associate Professor of Biology, University of Pittsburgh.
Wolf, Opal M., Assistant Professor of Biology, Goucher College.
Woodruff, L. L., Professor of Protozoölogy, Yale University.
Yntema, C. L., Assistant Professor, Cornell University Medical College.
Young, Roger A., General Education Board Fellow in Zoölogy, University of Pennsylvania.
Young, William C., Associate Professor of Primate Biology, Yale University.

Beginning Investigators, 1939

Algire, Glenn H., Weaver Research Fellow, University of Maryland, School of Medicine.
Alsup, Fred W., University of Pennsylvania.
Andelman, Sumner Y., Student, University of Buffalo, School of Medicine.
Arena, Julio F. De La, Auxiliary Professor of Biology, Universidad de la Habana.
Auringer, Jack, 1945 Burlingame, Detroit, Michigan.
Ballentine, Robert, Graduate Student, Princeton University.
Barden, Robert B., Graduate Teaching Assistant, Stanford University.
Belda, Walter H., Graduate Student in Zoölogy, Johns Hopkins University.
Black, Maurice, New York University.
Burnett, J. M., Graduate Student, Washington University.
Castle, Ruth M., Assistant in Zoölogy, Vassar College.
Churney, Leon, Instructor in Zoölogy, University of Pennsylvania.
Cohen, Irving, Research Assistant, Memorial Hospital, New York City.
Davenport, D., Instructor in Biology, Reed College.
Desreux, Victor, Graduate Fellow, Belgian American Foundation.
Donnellon, James A., University of Pennsylvania.
Dowling, Delphine L., Assistant in Botany, Vassar College.
Dumm, Mary E., Graduate Assistant, Bryn Mawr College.
Edds, Mac V., Assistant in Biology, Amherst College.
Fulton, George P., Teaching Fellow in Biology, Boston University.
Gilbert, William J., Graduate Assistant in Botany, University of Michigan.
Goulding, Helen J., Student, University of Toronto.
Gustafson, A. H., Assistant Professor, Williams College.
Hairston, Nelson G., Graduate Assistant, University of North Carolina.
Hall, Thomas S., Graduate Student, Yale University.
Harris, Daniel L., Instructor, University of Pennsylvania.
Hayashi, Teru, Research Student, University of Pennsylvania.
Hinchey, M. Catherine, Graduate Student, University of Pennsylvania.
Hollingsworth, Josephine, Graduate Student, University of Pennsylvania.
Horn, Edward C., Graduate Student, Princeton University.
Jones, Richard J., Student Assistant, University of Buffalo, School of Medicine.
Kobler, Jason S., Laboratory Assistant in Biology, Amherst College.
Lee, Lawrence, University of Rochester Medical School.
Levine, Harry P., Instructor in Zoölogy, University of Vermont.
Lipman, Harry J., Graduate Assistant, University of Pittsburgh.
Ludwig, Francis W., Graduate Student, University of Pennsylvania.
Merry, James, Cole Fellow in Botany, University of Michigan.
Moore, Betty C., Graduate Student, Columbia University.

Moore, John A., Assistant in Zoölogy, Columbia University.
Norris, Charles H., Graduate Student, Princeton University.
Ormsbee, Richard A., Assistant in Biology, Brown University.
Philips, Frederick S., Graduate Teaching Assistant, University of Rochester.
Pirenne, Maurice H., C. R. B. Graduate Fellow, Princeton University.
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Reinstein, Cecil R., Research Assistant, Emory University.
Reynolds, Donald M., Undergraduate Student, Harvard University.
Rose, S. Meryl, Teaching Assistant, Columbia University.
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Smith, Carl C., Fellow and Assistant in Biochemistry, University of Cincinnati.
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Urie, John C., Graduate Student, Villanova College.
Von Dach, Herman, Assistant, Zoölogy and Entomology, Ohio State University.
Wherry, John W., Graduate Assistant and Tutorial Fellow, Northwestern University.
Wilbur, Karl M., Research Fellow, University of Pennsylvania.
Wilde, Charles E., Jr., Student, Dartmouth College.
Woolley, John P., Zoölogy Assistant, Columbia University.
Zwilling, Edgar, Teaching Assistant, Columbia University.

Research Assistants, 1939

Alley, Armine, Demonstrator, McGill University.
Armstrong, Charles W. J., Demonstrator in Biology, University of Toronto.
Badger, Elizabeth A., Research Assistant, University of Cincinnati.
Baker, Linville A., Laboratory Assistant, Lilly Research Laboratories.
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Beam, C. A., Student, Brown University.
Bender, Joseph C., Laboratory Assistant, Swarthmore College.
Bilka, Paul J., Trinity College.
Bloch, Robert, Research Assistant, Columbia University.
Bowen, William J., Bruce Fellow, Johns Hopkins University.
Briscoe, Priscilla M., Research Assistant, University of Pennsylvania.
Brownell, K. A., Research Assistant, Ohio State University.
Butts, C. Earl, University of Maryland.
Calabrisi, Paul, Fellow in Anatomy, George Washington University.
Campbell, John B. S., Research Assistant, University of Pennsylvania.
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Costello, Helen M., Fellow by Courtesy in Zoölogy, University of North Carolina.
Crawford, John D., Alumnus, Milton Academy.
Crowell, Villa Bailey, Miami University.
Curtis, Howard J., Rockefeller Fellow, Johns Hopkins University Medical School.
Dewey, Virginia C., Research Assistant, Brown University.
Dowding, Grace L., University of Maryland, School of Medicine.
Dytche, Maryon, Graduate Student, University of Pittsburgh.
Dziemian, Arthur J., Graduate Student, Princeton University.

Ederstrom, Helge E., Assistant, Northwestern University.
Edman, Marjorie, Research Assistant, University of Cincinnati.
Erlanger, Margaret, Instructor, West Virginia University.
Finkel, Asher J., Research Assistant, The University of Chicago.
Foster, Richard W., Alumnus, Milton Academy.
Fraser, Doris A., University of Pennsylvania.
Furth, Olga B., Cornell University Medical College.
Gaines, Elizabeth, Research Assistant, Amherst College.
Genther, Theodore S., Assistant, University of Cincinnati.
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Grave, Caswell, II, Assistant, Washington University.
Grinnell, Stuart W., Research Associate, Swarthmore College.
Hendricks, Elliott M., Assistant, University of Cincinnati.
Höber, Josephine, University of Pennsylvania.
Howe, John H., Student, Brown University.
Hutchens, John, Laboratory Assistant, Lilly Research Laboratories.
Idoine, Leon S., Assistant in Biology, University of Toledo.
Kaliss, Nathan, Research Assistant, University of Pennsylvania.
Keefe, Eugene L., Research Assistant, Washington University.
Kleinholz, L. H., Harvard University.
Landis, Robert E., Graduate Student in Physiology, University of Pittsburgh.
Love, Genevieve, Brookville, Pennsylvania.
Mapp, Frederick E., Student, Atlanta University.
Martin, Rosemary D. C., Assistant, University of Toronto.
Maxfield, Mary E., Assistant Instructor, University of Pennsylvania.
Maxwell, Thomas, Jr., Student, University of Pennsylvania, School of Medicine.
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Musser, Ruth E., Student, Goucher College.
Naumann, Rudolph V., Fellow in Physiology, New York University, College of Medicine.
Netsky, Martin, Research Assistant, University of Pennsylvania.
Papale, Victoria L., Graduate Assistant in Biology, Montclair State Teachers College.
Papandrea, D. A., Student, Albany Medical College.
Pemberton, Frank A., Jr., Harvard University.
Phelps, Lillian A., Milbury, New Jersey.
Pool, Judith G. de Sola, Graduate Student, The University of Chicago.
Pool, Naomi de Sola, Student, Goucher College.
Ramsdell, Pauline A., Student, Swarthmore College.
Ricca, R. A., Student, University of Pennsylvania, School of Medicine.
Safford, Virginia, Assistant, Swarthmore College.
Schaeffer, Olive K., Research Assistant, Biological Institute, Philadelphia.
Scudamore, Harold H., Assistant, Northwestern University.
Snedecor, George W., Director Statistical Laboratory, Iowa State College.
Sommers, John H., Minneapolis, Minnesota.
Stonestreet, Garth A., Milton Academy.
Thompson, Ray K., Research Assistant, University of Maryland.
Trombetta, Vivian V., Assistant in Botany, Columbia University.
Weaver, Harry L., Research Assistant, Columbia University.
Wightman, John C., Science Instructor, Darrow School, New Lebanon, New York.

ZIMMERMAN, ALICE C., Assistant, Brown University.
ZWILLING, RHEA LYON, University of Maryland, School of Medicine.

Students, 1939

BOTANY

BOOTH, BEATRICE L., Student, Smith College.
DENMAN, THOMAS H., Student Assistant, Drew University.
FAIRCHILD, ROBERT S., Graduate Fellow, Iowa State College.
HANSON, ANNE M., Hunter College.
MYERS, DORIS U., Student, Barnard College.
PAGE, ROBERT M., Harvard University.
REYNOLDS, DONALD M., Student, Harvard University.
THIVY, FRANCESCA, Lecturer in Botany, Women's Christian College, Madras, India.
WHITESIDE, JULIA, Student, Wellesley College.

EMBRYOLOGY

BARDEN, ROBERT B., Graduate Student, Teaching Assistant, Stanford University.
BARKLEY, MABEL A., Teacher of Biology, Washington Public Schools.
BARNES, MARTHA R., Assistant Instructor, University of Illinois.
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BRANDAU, LOUISE B., Goucher College.
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BUEKER, ELMER D., Student, Washington University.
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NEVIN, FLOYD R., Instructor in Biology, Cornell University.
REINSTEIN, CECIL R., Research Assistant, Emory University.
WAKSMAN, BYRON H., Student, Swarthmore College.
WALTHER, JEANNE P., Wheaton College.
YOLLES, STANLEY, Brooklyn College.

INVERTEBRATE ZOOLOGY

ALLEN, BARBARA, Graduate Assistant, Mount Holyoke College.
BACON, ROBERT L., Student, Hamilton College.
BARTLETT, LAWRENCE M., Massachusetts State College.
BRADLEY, FREDERICK, Howard University.
BROWNE, SARAH D., Student, Pennsylvania College for Women.
BUEKER, ELMER D., Graduate Student, Washington University.
CAVENDER, JOHN C., Assistant in Biology, Brothers College of Drew University.
CHAMBERS, GLADYS M., Assistant Professor of Biology, Tougaloo College.
CHRISTIANSEN, GERTRUDE M., Student, Wilson College.
DAY, ELIZABETH, Student, Elmira College.
DOUGLAS, PETER L., Student, Harvard University.
DUBOIS, REBECKAH, Student, Vassar College.
EGAN, RICHARD W., Undergraduate Assistant in Biology, Canisius College.
EHRMANN, IRENE, Student, State Teachers College at Montclair.
FALES, CATHERINE H., Graduate Assistant, Mount Holyoke College.
FRANK, SYLVIA R., Graduate Fellow, Columbia University.
GRIMM, MADELON R., State Teachers College, Montclair.
HALL, EVELYN J., Mount Holyoke College.
HALL, MARY N., Student Assistant in Zoölogy, Connecticut College for Women.
HARRISON, ROBERT W., Oberlin College.
HEMPHILL, CATHERINE D., Bryn Mawr College.

Ifft, John D., Assistant, Yale University.
Jenkins, Lucille O., Student, Middlebury College.
Kidder, Anne M. M., Student, Bryn Mawr College.
Kincaid, James H., Biology Instructor, Birmingham Southern College.
Lininger, Richard E., Student, DePauw University.
Lower, George G., Teacher of Biology and General Science, Westtown Friends School.
Ludwig, Francis W., University of Pennsylvania.
McCalla, Frances L., Instructor, Agnes Scott College.
Mandrey, Jeanette L., Graduate Assistant, Wellesley College.
Marzulli, Francis N., Johns Hopkins University.
McDonald, Malcolm E., Graduate Assistant in Zoölogy, State University of Iowa.
Metz, Charles B., Student, Johns Hopkins University.
Molnar, George W., Graduate Student, Yale University.
Morris, Anne, Student, Elmira College.
Opton, Edward M., Graduate Student, Yale University.
Powers, Edward L., Jr., Student Assistant, Johns Hopkins University.
Ramsdell, Pauline A., Student, Swarthmore College.
Rayburn, Ruth, Student, Oberlin College.
Robertson, G. Gordon, Graduate Student, Assistant, Yale University.
Rogick, Mary D., Professor of Biology, College of New Rochelle.
Roller, Kathryn L., Graduate Student, Rutgers University.
Samorodin, A. J., Graduate Student, University of Minnesota.
Schneiderman, Frances H., Student, Hunter College.
Spence, Frances L., Student, Oberlin College.
Sweibel, Vivian, Hunter College.
Terry, Robert L., Earlham College.
Tonks, Robert E., Chairman, Department of Science, St. Andrew's School.
Voter, Muriel A., Instructor, Wheaton College.
Walker, Theodore J., Museum Assistant, University of Oklahoma.
Williams, Carroll M., Austin Teaching Fellow in Anatomy, Harvard University.
Williams, Lucy F., Radcliffe College.
Winborn, Morris K., Assistant, Amherst College.
Winsten, Alwin, 1823 Marmion Avenue, New York City, New York.
Zabelin, B. Elizabeth, Teacher of Biology, Chicago High School.

5. A TABULAR VIEW OF ATTENDANCE, 1935–1939

	1935	1936	1937	1938	1939
Investigators—Total	315	359	391	380	352
Independent	208	226	256	246	213
Under Instruction	56	76	74	53	60
Research Assistants	51	57	61	81	79
Students—Total	130	138	133	132	133
Zoölogy	55	55	57	54	55
Protozoölogy	16	17	16	10	12
Embryology	33	34	35	34	36
Physiology	20	22	16	22	21
Botany	6	10	9	12	9
Total Attendance	445	497	524	512	485
Less Persons Registered as Both Students and Investigators	16	24	13	16	14
	429	473	511	496	471

Institutions Represented—Total	143	158	165	151	162
By Investigators	111	120	134	125	132
By Students	70	77	79	67	72
Schools and Academies Represented					
By Investigators	—	2	3	4	2
By Students	3	3	2	1	2
Foreign Institutions Represented					
By Investigators	7	9	16	14	8
By Students	1	5	—	3	1

6. SUBSCRIBING AND COÖPERATING INSTITUTIONS IN 1939

Amherst College
Atlanta University
Belgian American Educational Foundation
Bowdoin College
Brothers College of Drew University
Brown University
Bryn Mawr College
California Institute of Technology
Canisius College
Carnegie Institute of Washington
College of Physicians and Surgeons
College of William and Mary
Columbia University
Connecticut College for Women
Cornell University Medical College
Dalhousie University
Dartmouth College
DePauw University
Duke University
Elmira College
Goucher College
Hamilton College
Harvard University
Harvard University Medical School
Howard University
Hunter College
Industrial and Engineering Chemistry, American Chemical Society
Iowa State College
Johns Hopkins University
Johns Hopkins University Medical School
Eli Lilly & Co.
Long Island University
McGill University
Massachusetts State College
Memorial Hospital, New York City
Morehouse College
Mount Holyoke College
Mundelein College
New York State Department of Health
New York University, Washington Square College
New York University
Northwestern University
Oberlin College
Pennsylvania College for Women
Princeton University
Purdue University
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
St. Francis Seminary
St. Johns College
Smith College
State University of Iowa
Syracuse University
Temple University
Tufts College
Union College
University of Buffalo School of Medicine
University of Chicago
University of Cincinnati
University of Illinois
University of Maryland Medical School
University of Missouri
University of Notre Dame
University of Pennsylvania
University of Pennsylvania School of Medicine
University of Pittsburgh
University of Rochester
University of Toledo
University of Virginia
Vanderbilt University Medical School
Vassar College
Villanova College
Washington University
Washington University Medical School
Wellesley College
Wesleyan University
Western Reserve University
Wheaton College
Williams College
Wilson College
Yale University

7. EVENING LECTURES, 1939

Friday, June 23
Mr. John S. Garth ..... "Animal Life in Equatorial Pacific."

Friday, June 30
Dr. Harold H. Plough ..... "The Influence of Temperature in Evolution as Shown by Genetic Studies on Drosophila."

Friday, July 7
Dr. Gerhard Fankhauser ..... "Polyploidy in Amphibians."

Friday, July 14
Dr. Rudolf Höber ..... "Cellular Activity Released by Organic Compounds in Surface Reactions."

Friday, July 21
Dr. T. M. Sonneborn ..... "Sexuality and Related Problems in Paramecium."

Friday, July 28
Dr. Viktor Hamburger ..... "Correlations between Nervous and Non-nervous Structure during Development."

Friday, August 4
Dr. G. K. Noble ..... "Neural Basis of Social Behavior in Vertebrates."

Friday, August 11
Dr. George Wald ..... "Vitamin A and Vision."

Thursday, August 17
Dr. J. D. Bernal ..... "The Biological Significance of Protein Structure."

Friday, August 18
Dr. Joseph Needham ..... "The Metabolism of the Gastrula, with Reference to the Amphibian Primary Organizer."

Monday, August 21
Dr. Robert Chambers ..... "Micromanipulative Studies."
Dr. C. C. Speidel ..... "Living Cells in Action."

Thursday, August 24
Dr. Franz Weidenreich ..... "Pithecanthropus and Sinanthropus, the Most Primitive Types of Fossil Man, and Their Relation to the Later Stages of Human Evolution."

Friday, August 25
Dr. E. Sinnott ..... "The Relation of Cells to Organs in Plant Development."

Friday, September 1
Mr. Duncan M. Hodgson ..... "Congo Color."

8. SHORTER SCIENTIFIC PAPERS, 1939

Wednesday, July 5

Dr. George W. Kidder ..... "The Effect of Biologically Conditioned Medium on the Growth of Colpidium campylum."

Dr. John Hutchens ..... "Respiration in Chilomonas paramecium."

Dr. Jay A. Smith ..... "Temperature and Starch and Fat in Chilomonas paramecium."

Tuesday, July 11

Dr. Floyd Moser ..... "The Differentiation of Isolated Rudiments of the Amblystoma punctatum Embryo."

Mr. Robert H. Silber ..... "The Production of Duplicatas Cruciata and Multiple Heads by Regeneration in Planaria."

Dr. L. G. Barth ..... "Neural Differentiation without Organizer."

Tuesday, July 18

Dr. Kenneth C. Fisher ..... "The Effect of Substrate Concentration on the Cyanide Sensitivity of the Oxygen Consumption of Yeast."

Mr. C. W. J. Armstrong ..... "A Comparison of Cyanide and Azide as Inhibitors of Cell Respiration."

Dr. Laurence Irving ..... "The Relation of Blood to Respiratory Ability of Fresh Water Fish."

Tuesday, July 25

Dr. David R. Goddard ..... "The Relation between Fermentation and Respiration in Higher Plants."

Dr. Claude E. ZoBell ..... "The Rôle of Bacteria in the Fouling of Submerged Surfaces."

Dr. E. W. Sinnott ..... "Cell Division and Differentiation in Living Plant Meristems."

Tuesday, August 1

Dr. Leonard I. Katzin ..... "The Ionic Permeability of Frog Skin as Determined with the Aid of Radioactive Indicators."

Dr. Kenneth Bailey ..... "Crystallization of Myogen from Skeletal Muscle."

Dr. Theodor von Brand ..... "Chemical and Histochemical Observations on Macracanthorhynchus hirudinaceus."

Mr. C. L. Claff and
Dr. G. W. Kidder ..... "pH Reactions during Feeding in the Ciliate Bresslaua."

Tuesday, August 8

Dr. J. D. Ferry ..... "The Dielectric Properties of Insulin Solutions."

Dr. J. A. Kitching ..... "The Effects of Lack of Oxygen and of Low Oxygen Tensions on the Activities of Some Protozoa."

Dr. Herbert Shapiro ..... "Nerve Asphyxiation and Aerobic Recovery in Relation to Temperature."

Dr. D. A. Marsland ..... "Effects of Hydrostatic Pressure upon Certain Cellular Processes."

Tuesday, August 15

Dr. Grace Townsend ..... "On the Nature of the Material from Fertilizable Nereis Eggs Inducing Spawning of the Male."

Dr. W. C. Young ..... "Ovum and Spermatozoön Age at the Time of Fertilization and the Course of Gestation and Development in the Guinea Pig."

Dr. Cornelius T. Kaylor ..... "Experiments on the Production of Haploid Salamanders."

Dr. A. B. Novikoff ..... "Regulation in Mosaic Eggs."

Tuesday, August 22

Dr. R. M. Cable and
Dr. A. V. Hunninen ..... "Studies on the Life History of Spelotrema Nicolli."

Dr. Rita Guttman ..... "Stabilizing Action of Alkaline Earths upon Crab Nerve Membranes, as Manifested in Resting Potential Measurements."

Mr. D. L. Harris ..... "An Experimental Study of the Pigment Granules of the Arbacia Egg."

Dr. L. V. Heilbrunn ..... "The Action of Calcium on Muscle Protoplasm."

9. THE GENERAL SCIENTIFIC MEETINGS, 1939

Tuesday, August 29

Dr. D. P. Costello and
Miss R. A. Young ..... "The Mechanism of Membrane Elevation in the Egg of Nereis."

Mr. Edgar Zwilling ..... "Determination and Induction of the Anuran Olfactory Organ."

Dr. Ethel Browne Harvey ..... "A Method of Determining the Sex of Arbacia, and a New Method of Producing Twins, Triplets and Quadruplets."

Dr. Ethel Browne Harvey ..... "An Artificial Nucleus in a Non-nucleate Half-egg."

Dr. G. H. Parker ..... "Color Responses of Catfishes with Single Eyes."

Dr. Grace Townsend ..... "A Vibration Sense in a Swarming Annelid."

Dr. D. H. Wenrich ..... "Food Habits of Endamoeba muris."

Dr. J. E. Kindred ..... "A Quantitative Study of the Hemapoietic Organs of Young Albino Rats."

Dr. William H. F. Addison ..... "On the Histology of the Mammalian Carotid Sinus."

Dr. Alexander Sandow ..... "On Clark's Theory of Muscular Contraction."

Dr. A. Navez and
Mr. John D. Crawford ..... "Conditions Governing the Frequency of Contraction of the Heart of Venus mercenaria."

Dr. F. J. M. Sichel ..... "The Refractory Period in the Non-conducted Response of Striated Muscle."

Dr. Alice M. Russell ..... "Pigment Inheritance in the Fundulus-Scomber Hybrid."

Miss Virginia Safford ..... "The Use of the Swimbladder by Fish in Respiratory Stress."

Dr. Herbert Shapiro ..... "Water Permeability of Chaetopterus Eggs."

Dr. M. H. Jacobs and
Dr. A. K. Parpart ..... "A Mechanism of Increased Cell Permeability Resembling Catalysis."

Dr. M. E. Krahl,
Miss A. K. Keltch and
Dr. G. H. A. Clowes ..... "Oxygen Consumption and Cell Division of Fertilized Arbacia Eggs in the Presence of Respiratory Inhibitors."

Mr. M. G. Netsky and
Dr. M. H. Jacobs ..... "Some Factors Affecting the Rate of Hemolysis of the Mammalian Erythrocyte by N-butyl Alcohol."

Mr. J. B. S. Campbell and
Dr. M. H. Jacobs ..... "Studies on the Permeability-decreasing Effect of Alcohols and Pharmacologically Related Compounds on the Human Erythrocyte."

Dr. Richard G. Abell ..... "Quantitative Studies of the Rate of Passage of Protein and Other Nitrogenous Substances through the Walls of Growing and of Differentiated Mammalian Blood Capillaries."

Wednesday, August 30

Dr. Eric G. Ball and
Miss Bettina Meyerhof ..... "The Occurrence of Cytochrome and Other Haemochromogens in Certain Marine Forms."

Mr. Carl C. Smith and
Mr. David Glick ..... "Some Observations on Cholinesterase in Invertebrates."

Dr. Kenneth Bailey ..... "Crystalline Myogen."

Dr. J. O. Hutchens and
Dr. M. E. Krahl ..... "Effect of Increased Intracellular pH on the Physiological Action of Substituted Phenols."

Dr. Albert E. Navez ..... "Fatty Acid Compounds in the Unfertilized Egg of Arbacia punctulata."

Dr. Aurin M. Chase ..... "Color Changes in Luciferin Solutions."

Mr. Fred W. Alsup ..... "Photodynamic Action in the Eggs of Nereis limbata."

Mr. Irving Cohen ..... "Cleavage Delay in Arbacia Punctulata Eggs Irradiated While Closely Packed in Capillary Tubes."

Dr. Rubert S. Anderson ..... "The X-Ray Effect on the Cleavage Time of Arbacia Eggs in the Absence of Oxygen."

Dr. P. S. Hensiiaw ..... "Fixation of X-Ray Effect by Fertilization in Arbacia Eggs."

Dr. William R. Duryee ..... "Does the Action of X-Rays on the Nucleus Depend upon the Cytoplasm?"

PAPERS READ BY TITLE

Mrs. R. K. Abramowitz and
Dr. A. A. Abramowitz ..... "Moulting and Viability after Removal of the Eyestalks in Uca pugilator."

Dr. A. A. Abramowitz ..... "A New Method for the Assay of Intermedin."

Miss Florence Armstrong,
Miss Mary Maxfield,
Dr. C. Ladd Prosser and
Mr. Gordon Schoepfle ..... "Analysis of the Electrical Discharge from the Cardiac Ganglion of Limulus."

Mr. Robert Ballentine ..... "The Intra-cellular Distribution of Reducing Systems in the Arbacia Egg."

Dr. H. W. Beams and
Dr. T. C. Evans ..... "Some Effects of Colchicine upon the First Division of the Eggs of Arbacia punctulata."
Dr. E. Frances Botsford ..... "Temporal Summation in Neuromuscular Responses of the Earthworm, Lumbricus terrestris."
Dr. F. A. Brown, Jr. ..... "The Source of Chromatophorotropic Hormones in Crustacean Eyestalks."
Dr. F. A. Brown, Jr. and
Mr. H. H. Scudamore ..... "Comparative Effects of Sinus Gland Extracts of Different Crustaceans on Two Chromatophore Types."
Dr. F. A. Brown, Jr. and
Mr. H. E. Ederstrom ..... "On the Control of the Dark Chromatophores of Crago Telson and Uropods."
Dr. John B. Buck ..... "Micromanipulation of Salivary Gland Chromosomes."
Dr. T. C. Evans and
Dr. H. W. Beams ..... "Effects of Roentgen Radiation on Certain Phenomena Related to Cleavage in Arbacia Eggs (Arbacia punctulata)."
Dr. John D. Ferry ..... "Chemical and Mechanical Properties of Two Animal Jellies."
Miss Judith E. Graham and
Dr. F. J. M. Sichel ..... "Response of Frog Striated Muscle to $CaCl_2$."
Dr. Charlotte Haywood ..... "The Permeability of the Toadfish Liver to Inulin."
Miss Josephine Hollingsworth . "Activation of Cumingia and Arbacia Eggs by Bivalent Cations."
Dr. Dwight L. Hopkins ..... "The Vacuole Systems of a Fresh Water Limacine Amoeba."
Dr. Cornelius T. Kaylor ..... "Cytological Studies on Androgenetic Embryos of Triturus viridescens Which Have Ceased Development."
Dr. Valy Menkin ..... "Effect of Leukotaxine on Cellular Permeability to Water."
Dr. Valy Menkin ..... "Effect of Leukotaxine on Cell Cleavage."
Dr. Floyd Moser and
Dr. J. A. Kitching ..... "Response of the Arbacia Egg Cortex to Chemical and Physical Agents in the Absence of Oxygen."
Dr. S. Milton Nabrit ..... "Further Studies on Regeneration in Fundulus Embryos."

Dr. A. J. Waterman ..... "The Action of Certain Drugs on the Intact Heart of the Compound Ascidian, Perophora viridis."

Dr. Opal Wolf ..... "An Effect of the Injection of a Solution of Dihydroxyestrin into Castrated Female Frogs, Rana pipiens."

DEMONSTRATIONS

Wednesday, August 30

Miss Bettina Meyerhof and
Dr. E. G. Ball ..... "The Cytochrome Spectra in Squid Heart."

Dr. Ethel Browne Harvey . (a) "Serial Photographs of Normal Development of Arbacia."
(b) "New Photographs of Parthenogenetic Merogones of Arbacia."
(c) "Twins, Triplets, and Quadruplets of Arbacia."
(d) "Stratified Echinoderm Eggs."

Dr. William H. F. Addison ..... "Photographs and Drawings Illustrating the Normal Histology of the Albino Rat."

Mr. C. Lloyd Claff ..... "A Migration-Dilution Apparatus for Sterilization of Protozoa."

Dr. R. L. Carpenter and
Mr. H. R. Sweet ..... "Dissection of Eyes and Brain of a 350-Pound Swordfish."

Mr. C. C. Smith ..... "The Use of the Clam Heart as a Test Object for Cholinergic Drugs."

Dr. Kenneth Bailey ..... "Myogen Crystals."

Dr. E. R. Clark and
Mrs. E. L. Clark ..... "Transparent Chambers Installed in Rabbit's Ears."
(a) "An R. G. Williams Transplant Chamber."

Dr. R. G. Abell ..... (b) "A New Type of Filter Disc Chamber for Collection of Capillary Filtrate."

Dr. Grace Townsend ..... "The Vibration Sense in Nereis limbata."

Dr. E. A. Wolf and
Miss Maryon M. Dytche ..... "Apparatus for Blood Calorimetry."

10. MEMBERS OF THE CORPORATION, 1939

1. Life Members

Allis, Mr. E. P., Jr., Palais Carnoles, Menton, France.
Andrews, Mrs. Gwendolen Foulke, Baltimore, Maryland.

Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts.
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania.
Conklin, Prof. Edwin G., Princeton University, Princeton, New Jersey.
Evans, Mrs. Glendower, 12 Otis Place, Boston, Massachusetts.
Foot, Miss Katherine, Care of Morgan Harjes Cie, Paris, France.
Gardiner, Mrs. E. G., Woods Hole, Massachusetts.
Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts.
Jackson, Mr. Chas. C., 24 Congress Street, Boston, Massachusetts.
King, Mr. Chas. A.
Lee, Mrs. Frederic S., 279 Madison Avenue, New York City, New York.
Lee, Prof. F. S., College of Physicians and Surgeons, New York City, New York.
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland.
Lowell, Mr. A. L., 17 Quincy Street, Cambridge, Massachusetts.
Means, Dr. J. H., 15 Chestnut Street, Boston, Massachusetts.
Moore, Dr. George T., Missouri Botanical Gardens, St. Louis, Missouri.
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Morgan, Mrs. T. H., Pasadena, California.
Morrill, Dr. A. D., Hamilton College, Clinton, New York.
Neal, Prof. H. V., Tufts College, Tufts College, Massachusetts.
Noyes, Miss Eva J.
Porter, Dr. H. C., University of Pennsylvania, Philadelphia, Pennsylvania.
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Shedd, Mr. E. A.
Thorndike, Dr. Edward L., Teachers College, Columbia University, New York City, New York.
Treadwell, Prof. A. L., Vassar College, Poughkeepsie, New York.
Trelease, Prof. William, University of Illinois, Urbana, Illinois.
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2. Regular Members

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Adams, Dr. A. Elizabeth, Mount Holyoke College, South Hadley, Massachusetts.

Addison, Dr. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.

Adolph, Dr. Edward F., University of Rochester Medical School, Rochester, New York.

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Baitsell, Dr. George A., Yale University, New Haven, Connecticut.

Baker, Dr. H. B., University of Pennsylvania, Philadelphia, Pennsylvania.

Ballard, Dr. William W., Dartmouth College, Hanover, New Hampshire.

Ball, Dr. Eric G., Johns Hopkins Medical School, Baltimore, Maryland.

Bard, Prof. Philip, Johns Hopkins Medical School, Baltimore, Maryland.

Barron, Dr. E. S. Guzman, Department of Medicine, The University of Chicago, Chicago, Illinois.

Barth, Dr. L. G., Department of Zoölogy, Columbia University, New York City, New York.

Beadle, Dr. G. W., School of Biological Sciences, Stanford University, California.

Beams, Dr. Harold W., Department of Zoölogy, State University of Iowa, Iowa City, Iowa.

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Behre, Dr. Elinor H., Louisiana State University, Baton Rouge, Louisiana.

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Bigelow, Prof. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.

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Bozler, Dr. Emil, Ohio State University, Columbus, Ohio.
Bradley, Prof. Harold C., University of Wisconsin, Madison, Wisconsin.
Bronfenbrenner, Dr. Jacques J., Department of Bacteriology, Washington University Medical School, St. Louis, Missouri.
Brooks, Dr. S. C., University of California, Berkeley, California.
Brown, Dr. Dugald E. S., New York University, College of Medicine, New York City, New York.
Buckingham, Miss Edith N., Sudbury, Massachusetts.
Buck, Dr. John B., Department of Zoölogy, University of Rochester, Rochester, New York.
Budington, Prof. R. A., Oberlin College, Oberlin, Ohio.
Bullington, Dr. W. E., Randolph-Macon College, Ashland, Virginia.
Bumpus, Prof. H. C., Duxbury, Massachusetts.
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Cannan, Prof. R. K., New York University College of Medicine, 477 First Avenue, New York City, New York.
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Cattell, Prof. J. McKeen, Garrison-on-Hudson, New York.
Cattell, Mr. Ware, Garrison-on-Hudson, New York.

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Cheney, Dr. Ralph H., Biology Department, Long Island University, Brooklyn, New York.
Chidester, Prof. F. E., Auburndale, Massachusetts.
Child, Prof. C. M., Jordan Hall, Stanford University, California.
Claff, Mr. C. Lloyd, Department of Biology, Brown University, Providence, Rhode Island.
Clark, Prof. E. R., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
Clark, Dr. Leonard B., Union College, Schenectady, New York.
Cleland, Prof. Ralph E., Indiana University, Bloomington, Indiana.
Clowes, Dr. G. H. A., Eli Lilly and Company, Indianapolis, Indiana.
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Crowell, Dr. P. S., Jr., Department of Zoölogy, Miami University, Oxford, Ohio.
Curtis, Dr. Maynie R., Crocker Laboratory, Columbia University, New York City, New York.
Curtis, Prof. W. C., University of Missouri, Columbia, Missouri.
Dan, Dr. Katsuma, Misaki Biological Station, Misaki, Japan.
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SEROLOGICAL REACTIONS AND SPECIES SPECIFICITY OF SOME HELMINTHS

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Introduction

Concepts regarding classification and relationships of organisms have been based chiefly on adult and developmental morphology, but the value of particular structures in determining descent has been variously interpreted by different authors. Accordingly, opinions concerning the phylogenetic status of certain forms have differed. Furthermore, failure to reach agreement as to what constitutes a species has provoked much discussion and controversy. The earlier studies dealing with morphology and development have been supplemented in recent years by the use of serological reactions. Long employed in the diagnosis and control of certain diseases, these reactions have been utilized also to determine species specificity and interrelationships of many organisms.

Serological methods have been increasingly stressed in experimental work designed to clarify phylogenetic relationships of parasitic worms and to test taxonomic systems based on morphological characters. Consideration of the several types of serological reactions indicated clearly that the precipitin test is best suited for the determination of species specificity and interrelationships of organisms. This test lends itself admirably to qualitative, quantitative, and *objective* interpretations, which are necessary and fundamental for the evaluation of serological relationships.

Historical Review

The precipitin reaction was discovered by Kraus (1897). He noted that blood sera of goats which had been inoculated with sterile cholera, typhoid or plague culture filtrates caused precipitates when mixed with their respective bacterial filtrates. The reaction was specific since only the filtrate of the bacterium used for immunization would produce a precipitate when mixed with the antiserum.

From the early researches of Bordet (1899) and of Tchistovitch (1899) it became evident that foreign proteins other than bacterial

ones were antigenic and could provoke the formation of precipitins when injected into an animal.

The discovery by Uhlenhuth (1901) that the precipitin reaction was not strictly specific, since an antiserum against one protein may react with other closely related proteins as well as with the protein used in its production, is the basis for the application of serological reactions to certain biological problems. He discovered that these reactions are quantitatively, as well as qualitatively, specific, i.e., any antiserum will react more strongly with the antigenic substance used in its formation (homologous reaction) than with other antigenically different, though closely related, substances (heterologous reactions), the degree of relationship being indicated by the strength of the heterologous reactions as compared to the homologous one.

Ascoli (1902), Graham-Smith and Sanger (1903), and Fornet and Müller (1908, 1910) modified Kraus' flocculation technique by introducing the antiserum into the precipitin tube below the antigen; at the zone of junction of the two a layer or "ring" of precipitate formed and the end-point or titer could be determined with greater precision.

Nuttall's (1904) extensive and outstanding study of several thousand qualitative and quantitative precipitin reactions with the blood of more than nine hundred species of animals confirmed, in general, the classification and relationships of these animals based on comparative anatomy and embryology.

A distinct advance was made by the work of Boyden (1926) who, employing Ascoli's "ring" technique, determined the titers and obtained confirmation of his quantitative reactions by reciprocal tests.

A. Aqueous Extracts in Serological Reactions

1. Alcohol-ether-soluble substances not removed

Research employing aqueous, helminthic antigens from which lipids had not been removed by preliminary alcohol-ether extraction has yielded interesting and suggestive, but variable and inconsistent, results. By injection of rabbits with increasing doses of a saline extract of *Taenia saginata*, Langer (1905) obtained antisera which yielded precipitates with both homologous and heterologous antigens.

Schwartz (1920) noted positive precipitin reactions of varying intensities between materials of *Ascaris*, *Belascaris*, *Toxascaris* and *Ascaridia*, depending on the degree of relationship of these members of the family Ascaridae (Ascaroidea). However, he obtained no precipitates between *Ascaris* antisera and the antigens of two genera, *Dictyocaulus* and *Strongylus*, belonging to the superfamily Strongyloidea.

Hektoen (1926) concluded that the precipitin reaction yields species-specific reactions and affords interrelationships which parallel those deduced from morphological comparisons of the cestodes and nematodes studied. The dilution factor for the homologous tests was variable, ranging from 1 : 400 to 1 : 10,000, but several of the *heterologous* reaction titers fell *within* this range.

Canning's (1929) report on precipitin reactions, employing various tissues of *Ascaris lumbricoides* and related ascarids, clearly indicated a serological differentiation of tissues of a single species as well as a variation in the reactions of homologous tissues from different species. Anti-tissue sera, the antigens of which were derived from a single germ layer, gave the strongest reaction with their homologous test antigen.

Eisenbrandt (1936, 1938) emphasized the importance of knowing the concentration of nitrogen in the extract, from which the protein content may be determined, but stated that "it cannot be said that the proteins are the only factors involved in these tests." Reciprocal tests confirmed the original ones in but a single case. Of further interest are the high titers which were obtained upon the injection of only 0.0096 gram of nitrogen (60 mg. of protein), and the extensive range of homologous titers which varied from 1 : 2,048,000 for *Toxocara canis* to 1 : 2000 for *Ascaridia lineata*, a range which included many heterologous titers.

Nitrogen-containing substances are not completely active, nor are they the only factor to be considered in serological reactions. Boyd and Hooker (1934) stated that "one has no assurance that all the antigenic 'nitrogen' is immunologically active." Viktoroff, Guintze-Verner and Demidova (1936) concluded that the precipitin titer of serum gives a more exact measure of its value than the amount of nitrogen in the precipitate. Levine and Moody (1939) stated that "Variation between species in total nitrogen content . . . does not form a significant variable." Antigens of greater nitrogen content did not produce antisera of *consistently* higher titer than that elicited by antigens of lower nitrogen content.

In addition to non-specific precipitation noted in reactions between lipid-containing antigens and immune sera, Eisenbrandt (1938) observed that "Sometimes the presence of lipids resulted in flocculation (of the antigen) even though the extract was sterile." Furthermore, lipids may cause precipitation even in normal sera, as reported by Mackie and Anderson (1937).

Group reactions have been noted also in complement fixation tests when lipid-containing, aqueous extracts were used as antigens. Chung

and T'ung (1939) concluded, on the basis of serological investigations involving hydatid and cysticercus cyst fluids, that complement fixation and intradermal tests are immunological group reactions to somatic cestode infections rather than specific reactions for the species involved in any particular case. The group specificity was not confined to cestodes or even to helminths, because the serum of kala-azar patients gave positive complement fixation reactions with hydatid cyst fluid; according to the authors, this positive reaction may be related to the high globulin content of the blood of kala-azar patients.

2. Alcohol-ether-soluble substances removed

In contrast to the variable and inconsistent results afforded by the use of whole-worm antigens, antisera produced by the injection of lipid-free antigenic materials tend to maintain species specificity when employed in serological reactions. Bachman (1928) and Augustine and Theiler (1932), after a preliminary extraction with ether to remove the lipoidal materials, extracted powdered *Trichinella spiralis* larvae with physiological salt solution. Specific, positive precipitin reactions in antigen dilutions of 1 : 2000 were obtained with antisera of injected animals. The latter authors reported positive intradermal reactions which paralleled those from the precipitin tests when antigen dilutions of 1 : 10,000 were used. In later reports, Bachman and Rodriquez-Molina (1932), using *Ascaris lumbricoides* and *Necator americanus* materials, recorded group-specific intradermal tests, but dilutions of only 1 : 200 were employed.

Stumberg (1930) performed precipitin and complement fixation tests with extracts of numerous helminths. Slightly higher titers were attained with the sera of rabbits injected with adult than with larval antigens, but the stages of the life cycle could hardly be considered serologically distinct. The antibodies produced in immunized rabbits were species-specific in antigen dilutions of 1 : 4000 or over, group-specific in dilutions from 1 : 1000 to 1 : 4000, and non-specific in dilutions below 1 : 1000.

It may be concluded from these data that species-specific serological reactions are more likely to result with antisera produced by the injection of lipid-free antigens than with those evoked by lipid-containing antigens.

B. Alcoholic Extracts in Serological Reactions

Extraction with alcohol yields antigens which are fundamentally lipoidal in nature. Lipids are unable to incite antibody formation by themselves but serve as haptens, since they can react with antisera produced by injection of lipid-protein mixtures. Landsteiner's studies

led him to the conclusion (1928), fundamental for all precipitin work, that a two-fold system of specificity exists in animals, the specificity of proteins and that of haptens. In the case of the proteins, the serological and chemical properties, in general, parallel closely the zoölogical relationships. On the other hand, reactions involving haptens are less perfectly correlated with the systematic position of species tested, since haptens of closely related species may be very different chemically and those of distantly related species very similar, if not identical. Manwaring (1934) reported that proteins of two distantly related species may be so completely homologized by artificial conjugation with the same hapten as to be readily mistaken for protein from the same species. Thus, lipids, being haptogenic substances and not *species*-specific in their reactions, could overshadow the species specificity of proteins, since antisera against such conjugates apparently contain both anti-protein and anti-hapten factors.

Group specificity has been obtained in complement fixation reactions when alcoholic extracts of trematodes have been employed as antigens. Fairley (1919–1933) noted group reactions with cercarial antigens of several species of *Schistosoma* and concluded, on the basis of complement fixation tests with lipid-containing materials, that there is an antigenic complex common to mammalian schistosomes. In contradistinction to Fairley, Cawston (1921) and Höppli (1921) reported group reactions between *Schistosoma* and *Fasciola hepatica* materials. Employing alcoholic cercarial antigen of *Schistosomatium pathlocopticum*, Tanabe (1923) obtained positive results with the antiserum of rats infected with this parasite as well as with sera of others harboring *S. japonicum*. In contrast to these results, species specificity was noted by Andrews (1935) for *S. japonicum* in complement fixation tests, *but* antisera of individuals infected with other species of *Schistosoma* were not tested.

Kolmer, Trist and Heist (1916) concluded that the complement fixation reaction is inadequate for differentiation of related species of cestodes, since the reactions indicated close relationship between the tapeworms, *Taenia serrata* (= *T. pisiformis*) and *Dipylidium caninum*, which are quite distinct morphologically. According to the authors, lipoidal substances present in the extracts were capable of absorbing complement even with luetic antisera. Wharton (1930, 1931) stated that tapeworm lipids are not true antigens but under certain conditions can influence the character of the antigenic proteins. The reaction of *Cysticercus pisiformis* antigen was relatively *weaker* with antiserum of its adult form, *T. pisiformis*, than with *Moniezia* antiserum, which indicates that antisera to lipid-containing antigens yield group-specific

reactions. Group specificity was recorded by Sievers (1935) for the tapeworms, *Bothriocephalus latus*, *Taenia saginata* and *Echinococcus*, but species-specific antigen components were found by quantitative methods of testing.

In experiments with nematodes, Kolmer, Trist, and Heist (1916) observed cross reactions indicative of close serological relationship between materials of *Ascaris canis* (=*Toxocara canis*) and *Strongylus gigas* (=*Dioctophyme renale*), species which are very different morphologically. Group reactions in the nematode family Filariidae were obtained by Fairley (1931), who used *Dirofilaria immitis* antigen to diagnose *Wuchereria bancrofti*, *Loa loa*, and *Onchocerca volvulus* infections by complement fixation methods.

Thus, it may be concluded that, in general, antigens prepared by alcoholic extraction of materials, and therefore composed basically of lipids, yield *group*-specific reactions. This group specificity was especially apparent when complement fixation technique was employed, and Neveu-Lemaire (1936) concluded that complement fixation tests yield no better than group specificity.

C. Purified Polysaccharides in Serological Reactions

Until recently, protein has been regarded as necessary for antibody formation and hence immunization, but it has been found that the polysaccharides may induce the formation of and react with antibodies. Though Zozaya (1932) obtained agglutinating and precipitating sera by injecting rabbits and horses with various polysaccharides adsorbed on collodion particles, Landsteiner and Jacobs (1932) failed to confirm this work. In earlier experiments, polysaccharides failed to stimulate the production of antibodies, because they had been altered considerably in the process of purification. Avery and Goebel (1933) reported that mice, though not rabbits, could be immunized by injections of natural acetylated, Type I pneumococcal polysaccharides, and the resulting immunity was type-specific. Heidelberger, Kendall and Scherp (1936) produced immunization to pneumococci by injections of protein-free preparations of their natural polysaccharides. Raistrick and Topley (1934) obtained antisera containing agglutinins and precipitins by the injection of a product from *Bacterium aertrycke* (=*Salmonella aertrycke*) which contained no demonstrable protein.

Purified polysaccharides of helminths have recently been shown to be antigenic independent of any protein. Using *Taenia taeniaformis*, Campbell (1936*a*) found that a polysaccharide fraction conferred a considerable degree of protection against infection with the onchospheres, though the proteins were more efficacious. Later he (1936*b*) reported that protein and purified polysaccharide fractions of *Ascaris*

lumbricoides were immunologically distinct in precipitin tests. Purified polysaccharides prepared by Campbell (1937) from a number of helminths were tested with whole-worm antisera by the precipitin reaction. Polysaccharide fractions exhibited a much greater specificity than the complex solutions of whole worms. The use of whole-worm antigens of the closely related forms, *Ascaris lumbricoides* and *A. suum*, indicated their specific identity, whereas polysaccharide fractions, when employed as antigens, showed differences between the two. It is evident, then, that purified polysaccharides of helminths can, even in the absence of protein, stimulate the formation of antibodies.

Species Specificity

Serological reactions have been employed rather extensively in studies on helminths, but divergent results have been reported by the several investigators. Langer (1905), working with *Taenia saginata*, *T. solium*, *T. cucumerina* (=*Dipylidium caninum*), and *Ascaris lumbricoides* materials, injected aqueous extracts which contained 500 mg. of protein, and he recorded a titer of 1 : 15,000 for species specificity. Bachman (1928) obtained a species-specific titer of 1 : 2000 upon administration of 240 to 500 mg. of *Trichinella spiralis* antigen. Canning (1929) injected only 40 mg. of dried, powdered *A. lumbricoides* antigen suspended in saline solution, but homologous titers varied from 1 : 1600 to 1 : 4000 in tests performed with antisera withdrawn from the rabbits ten to fifteen days after the last injection. Stumberg (1930) recorded species-specific titers of 1 : 4000 or greater upon the injection of 100 mg. of powdered cestode, nematode and acanthocephalan antigens. Wolfe (1935), working with mammalian blood sera, obtained a titer of 1 : 12,800 or greater for species specificity, but he administered 1.6 grams to 2.75 grams of protein by the long method of injection or 0.16 grams to 0.275 grams by the short method. Eisenbrandt (1938) reported a variation in the species-specific titers from 1 : 2000 to 1 : 2,048,000 with antisera produced by injection of cestode, nematode and acanthocephalan materials in aqueous solutions containing 60 mg. of protein. In experiments reported by Wolfe (1939), homologous titers varied from 1 : 16,000 to 1 : 1,024,000 when antisera produced by injection of mammalian, avian or reptilian sera were employed in precipitin tests. These high-titered antisera resulted from the administration of only 0.001 gram of protein per kilogram of body weight.

Moulton (1923) reported that chemical constancy or maturity in vertebrates is not established in the early stages of development. Manwaring (1934) stated that "marked changes in fractional specificities are also demonstrable in different stages of the normal life cycle of all plant, animal and microbic species thus far adequately studied."

Accordingly, the purposes of the present investigation are to determine: (1) the minimum amount of antigen necessary to provide reliable and constant precipitin reactions; (2) species-specific titers, thus giving an objective method for determining whether a form under consideration is a new species or merely a strain, variety or race of an established species; (3) the time in the life cycle at which species specificity is established, with a view to using serological methods in identifying the various larval with the adult stages of a parasite and thus aid in working life cycles; (4) interrelationships of the several species of trematodes and cestodes which were used; and (5) the value of the reciprocal tests for confirmation of the original results.

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Materials and Methods

Materials for establishing the time at which zoölogical specificity is attained have been supplied by several stages in the life cycles of the trematodes, *Parorchis acanthus*, *Cryptocotyle lingua* and *Zygocotyle lunata*, and the cestode, *Taenia taeniaformis*. In addition to these species, *Plagitura parva*, *Siphodera vinaledwardsii*, *Dipylidium caninum*, *Tetrabothrius* sp., and *Moniezia expansa* were used to determine the dilution limits for species specificity.

Preparation of Antigen

To free them of debris and foreign proteins, the worms, when obtained, were washed through several changes of physiological saline solution, filtered sea water, or distilled water, depending on the source of the parasite. Following this preliminary treatment, they were washed several times in sterile media and finally, if necessary, quickly through sterile distilled water to remove excess salts. Cotton-plugged sterile Florence flasks (liter-size), containing 500 cc. of wash solution, were used, and transfers of the worms were made by means of sterile pipettes. The parasites were transferred a minimum of six times, allowing half an hour or more in each flask, thus reducing the bacterial contamination to a minimum. During this washing procedure, the solutions which were used to wash worms from warm-blooded hosts were kept in the incubator at 37° C. Subsequent manipulations were done carefully to avoid contamination.

The living worms were frozen rapidly, using dry ice, and kept at this low temperature in a vacuum desiccator (residual pressure of 0.001

mm. Hg) over anhydrous calcium chloride until thoroughly dried. Rapid freezing preserves the original structure of labile substances, and, when the material has been thoroughly desiccated, it can be extracted with ether or absolute alcohol without destruction or denaturization of the proteins. The high vacuum causes rapid dehydration, and a certain amount of autorefrigeration would occur as a consequence of the rapid evaporation. Furthermore, this procedure removes almost all oxygen which could act on the tissue proteins and other antigenic substances to alter their native structure. Desiccation in the frozen state results in dried material which has a spongy texture and such material is more readily pulverized and more soluble than the flaky substances which result from dehydration at room temperature and atmospheric pressure.

After twenty-four hours in the vacuum, the worms were triturated, using an agate mortar and pestle, and then returned to the desiccator. Complete desiccation was indicated when the material arrived at a constant weight.

To remove the lipids, Bloor's mixture, which consists of three parts absolute ether to one part absolute alcohol, was employed. Extraction was done in Erlenmeyer flasks placed in a mechanical shaker oscillating at the rate of 220 times per minute. One part by weight of powdered worm material was mixed with one hundred parts by volume of extractant, and repeated extractions were made until the subsequent weight of the dry residue remained constant, at which time the material was assumed to be lipid-free.

After weighing the lipid-free residue, it was extracted for twenty-four hours (under constant agitation) with either physiological salt solution adjusted to a pH of 7.2–7.4 or with buffered saline solution maintaining a pH of 7.3. The pH of the test antigen solution must be approximately 7.2–7.4 to obviate the possibility of pseudo-positive reactions with antiserum.

After aqueous extraction the material was filtered through a sterilized Jenkins' bacteriological filter, and the sterile filtrate was transferred to sterile vaccine bottles. The residue was thoroughly desiccated and weighed. The exact amount of material, probably mainly protein and carbohydrate, in solution was determined by calculating the difference in weight of residues before and after the aqueous extraction. The clear, sterile filtrate was kept in the refrigerator until needed.

Antibody Production

For injection purposes, healthy rabbits which had been kept under observation several weeks prior to experimentation were used. All

injections were made into the lateral ear veins, since it was found that higher-titered antisera were produced by the intravenous than by the intraperitoneal route. Most satisfactory procedure involved four intravenous injections, one every third day, with gradually increasing dosage and with a ten-to-twelve day interval after the last injection before blood was withdrawn. This short method of antibody production in rabbits has certain advantages, which include the greater specificity of the antisera produced and the decrease in time involved in obtaining an antiserum. Furthermore, smaller amounts of antigen will yield antisera high in specificity for homologous antigen, whereas by the long method, in which large amounts have been used, the specificity may be masked, because the titers of heterologous reactions increase greatly while the homologous reaction titer becomes only slightly higher.

In the series of four injections, a total of 40 mg. of dry weight, lipid-free antigen was injected. The 40 mg. of antigen was divided into doses of increasing amounts, i.e., approximately 4, 8, 12 and 16 mg.

Ten to twelve days after the last injection, as much as 20 cc. of blood was withdrawn from the heart by a large, sterile hypodermic syringe fitted with a 19-gauge needle; the blood was placed in sterile, cotton-plugged centrifuge tubes and allowed to clot at room temperatures. Having clotted, it was placed in the refrigerator for several hours. Subsequent to centrifugation of the clotted blood, the serum was drawn off and used immediately for tests or placed in sterile tubes.

Precipitin Test

The usual technique for making the precipitin test was employed; 0.3 cc. of antiserum in a precipitin tube was overlaid by 0.3 cc. of variously diluted test antigen. The precipitin tubes were 4.0 mm. in diameter and were graduated in millimeters. Dilutions of the antigen were made with sterile, buffered saline solution maintaining a pH of 7.3. To make some of the precipitin tests it was necessary to evaporate the test solutions to the desired antigenic concentrations. Evaporation was effected by placing the extract in sterile Petri or crystallizing dishes in a partial vacuum over anhydrous calcium chloride. In the homologous reactions, antigenic concentrations of 1 : 1000, 1 : 2000, 1 : 3000, 1 : 4000, 1 : 6000, 1 : 8000 and 1 : 10,000 were used, and dilutions from 1 : 10 to 1 : 2000 were employed to determine the titers of the heterologous tests. In a positive reaction, a ring or layer of white precipitate occurred at the interphase of the serum and antigen. The width of the ring was dependent primarily on the strength of the antigen solution employed in making any particular test, provided antisera

of equivalent strengths were used. However, the width and density of the layer of precipitate were dependent secondarily on the degree of mixture which resulted when the antigen was placed over the serum.

The titer of a reaction is the highest antigen dilution which gives a visible precipitate when tested with an antiserum, either homologous

TABLE I

Homologous Tests to Determine Titers
(Each test performed in duplicate and many in triplicate)

Species	Temp.	1 : 1000	1 : 2000	1 : 3000	1 : 4000	1 : 6000	1 : 8000	1: 10,000
Parorchis Adult	Rm.	0	2.0 mm.	1.0 mm.	0.5 mm.	±	−	0
	37°C.	0	2.0 mm.	1.0 mm.	1.0 mm.	+	−	−
Parorchis Rediae	Rm.	0	2.0 mm.	1.0 mm.	1.0 mm.	+	−	0
	37°C.	0	3.0 mm.	1.5 mm.	1.0 mm.	0.5 mm.	−	−
Parorchis Metacercariae	Rm.	0	2.0 mm.	0	1.5 mm.	+	−	0
	37°C.	0	3.0 mm.	1.5 mm.	0.5 mm.	+	−	−
* *Cryptocotyle* Adult	Rm.	0	2.5 mm.	1.0 mm.	+	−	−	0
	37°C.	0	2.5 mm.	2.0 mm.	0.5 mm.	−	−	−
Cryptocotyle Cercariae	Rm.	0	2.0 mm.	1.0 mm.	0.5 mm.	+	−	0
	37°C.	0	2.5 mm.	2.0 mm.	1.0 mm.	0.5 mm.	−	−
Zygocotyle Adult	Rm.	0	1.5 mm.	0	0.5 mm.	+	−	0
	37°C.	0	2.0 mm.	1.0 mm.	0.5 mm.	+	−	−
Zygocotyle Metacercariae	Rm.	0	2.0 mm.	0	0.5 mm.	+	−	0
	37°C.	0	2.0 mm.	0	1.0 mm.	0.5 mm.	−	−
Siphodera Adult	Rm.	1.5 mm.	1.5 mm.	+	+	−	−	0
	37°C.	2.0 mm.	1.5 mm.	0.5 mm.	+	−	−	−
Plagitura Adult	Rm.	3.0 mm.	2.0 mm.	1.0 mm.	+	±?	−	0
	37°C.	3.0 mm.	2.0 mm.	1.5 mm.	0.5 mm.	+	−	−
Monieza Adult	Rm.	3.5 mm.	2.5 mm.	1.5 mm.	0.5 mm.	+	−	0
	37°C.	3.5 mm.	3.0 mm.	1.0 mm.	0.5 mm.	0.5 mm.	+(1)-(3)	−
Dipylidium Adult	Rm.	2.0 mm.	1.0 mm.	0	0.5 mm.	−	−	0
	37°C.	3.0 mm.	2.5 mm.	1.0 mm.	1.0 mm.	0.5 mm.	−(? in 2)	−
Tetrabothrius Adult	Rm.	3.0 mm.	2.0 mm.	0	0.5 mm.	+	−	0
	37°C.	3.5 mm.	2.0 mm.	1.0 mm.	0.5 mm.	+	−	−
T. taeniaformis Adult	Rm.	2.0 mm.	1.0 mm.	1.0 mm.	0.5 mm.	+	−	0
	37°C.	0	2.0 mm.	1.0 mm.	0.5 mm.	++	−	−
T. taeniaformis Cysticerci	Rm.	2.0 mm.	1.0 mm.	1.0 mm.	0.5 mm.	+	−	0
	37°C.	2.0 mm.	1.5 mm.	1.5 mm.	1.0 mm.	0.5 mm.	−	−

* Only 30 mg. of antigen injected.

or heterologous, the amount of antigen being determined on the basis of dry weight of antigenic material *actually in solution.*

In all cases the readings and titers were taken at the end of one hour, at room temperature in one series and at 37° C. in the other. As reported by Boyden (1926) and Eisenbrandt (1938), a period longer than one hour is unsatisfactory because the layer becomes diffuse and begins to settle out, resulting in poor determination of the end-point or titer.

Control tests were made, using (1) normal sera and antigen solutions, (2) immune sera and the extracting salt solution, and (3) normal sera and the extractant. "Normal serum" indicates the serum of the same rabbit as that used for immunization, the blood having been taken by intracardial puncture shortly before the series of injections was started.

Observations and Results

Tables were prepared to present the results of the experiments in graphic form. Table I shows the data from homologous precipitin tests performed at room temperatures and at 37° C. The width of the rings of precipitate was used to determine the effects of temperature variations on the reactions, and the titers of homologous reactions defined the end-point of a species-specific test. In the higher dilutions of the antigen, accurate measurement of the width of the ring in a positive reaction could not be made, and such tests were recorded by means of the plus sign (+); negative reactions were indicated by minus signs (−) and tests which were not performed, by zeros (0). Especially in the higher dilutions, positive reactions are more definite at 37°, and one must conclude that it is advisable to employ a constant high temperature (37° C.) in determination of titers of serological reactions.

Table II presents the titers of both homologous and heterologous reactions between the various species of trematodes, and Table III, similar data on the cestodes which were used in the experiments. The number in parenthesis indicates the number of tests from which an average was taken to denote the titer; the variation in the titers of individual tests was never more than plus or minus one dilution from the average. No reactions occurred between materials of the trematodes and cestodes which were tested. The limited amount of trematode antigens did not permit a comparison between heterologous reactions at different temperatures; therefore the tests, in which trematode material was used, were performed at the constant temperature of 37° C. With few exceptions, the titer which could be taken to be species-specific is 1 : 6000, but, in order that every case be included, it is necessary to assign a value of 1 : 4000 to species specificity. It may be noted that the titers of reactions are nearly the same when antigens prepared from various stages in the life cycle of a single species are tested with antisera produced by the injection of a single antigenic substance, either larval or adult. In cases of difference, however, the higher titer obtains in reactions with an antiserum tested against the same antigen which had been used in its formation. However, the

TABLE II

Homologous Titers Denoting Species Specificity
Heterologous Titers Denoting Relationships
Tests performed at 37° C.
(Number in parenthesis indicates number of tests)

Antiserum \ Antigen	*Parorchis* Adult	*Parorchis* Rediae	*Parorchis* Metacercariae	*Cryptocotyle* Adult	*Cryptocotyle* Cercariae	*Siphodera* Adult	*Plagitura* Adult	*Zygocotyle* Adult	*Zygocotyle* Metacercariae
Parorchis Adult	1 : 6000 (7)	1 : 4000 (8)	1 : 4000 (6)	1 : 600 (6)	1 : 400 (6)	1 : 250 (3)	1 : 60 (3)	1 : 10 (4)	1 : 10 (4)
Parorchis Rediae	1 : 4000 (8)	1 : 6000 (6)	1 : 6000 (6)	1 : 400 (8)	1 : 600 (10)	1 : 350 (3)	1 : 80 (3)	0 (10)	? (6) 1 :10(4)
Parorchis Metacercariae	1 : 4000 (8)	1 : 6000 (7)	1 : 6000 (4)	1 : 400 (7)	1 : 600 (6)	1 : 300 (3)	1 : 60 (3)	1 : 10 (3)	1 : 10 (5)
* *Cryptocotyle* Adult	1 : 400 (4)	1 : 400 (7)	1 : 400 (5)	1 : 4000 (6)	1 : 4000 (8)	1 : 1000 (7)	1 : 100 (3)	0	0
Cryptocotyle Cercariae	1 : 600 (5)	1 : 600 (7)	1 : 600 (6)	1 : 6000 (5)	1 : 6000 (6)	1 : 1200 (8)	1 : 150 (3)	0	0
Siphodera Adult	1 : 275 (7)	1 : 300 (9)	1 : 300 (7)	1 : 1200 (7)	1 : 1200 (4)	1 : 4000 (4)	1 : 30 (6)	0	0
Plagitura Adult	1 : 80 (4)	1 : 60 (4)	1 : 60 (3)	1 : 150 (6)	1 : 200 (6)	1 : 50 (7)	1 : 6000 (5)	0	0
Zygocotyle Adult	1 : 10 (4)	1 : 20 (6)	1 : 10 (5)	1 : 40 (3)	1 : 50 (3)	0	0	1 : 6000 (7)	1 : 4000 (7)
Zygocotyle Metacercariae	1 : 10 (5)	1:10(2) ?(3)	1 : 20 (6)	1 : 40 (3)	1 : 50 (4)	0	0	1 : 6000 (6)	1 : 6000 (6)

* Only 30 mg. of antigen injected.

TABLE III

Homologous Titers Denoting Species Specificity
Heterologous Titers Denoting Relationships
(Number in parenthesis indicates number of tests)

Antiserum \ Antigen	Temp.	*Moniezia* Adult	*Dipylidium* Adult	*T. taeniaformis* Adult	*T. taeniaformis* Cysticerci	*Tetrabothrius* Adult
Moniezia Adult	Rm.	1 :6000(5)	1 :400(6)	1 : 50(5)	1 : 50(5)	0
	37°C.	1 :6000(4)	1 :600(8)	0	1 : 70(4)	0
Dipylidium Adult	Rm.	1 :400(6)	1 :4000(4)	1 : 100(8)	1 : 100(7)	0
	37°C.	1 :400(6)	1 :6000(4)	1 : 150(7)	1 : 100(7)	0
T. taeniaformis Adult	Rm.	1 :50(7)	1 :100(4)	1 : 6000(6)	1 : 4000(6)	0
	37°C.	1 :50(7)	1 :100(4)	1 : 6000(4)	1 : 6000(4)	0
T. taeniaformis Cysticerci	Rm.	1 :60(5)	1 :100(5)	1 : 5000(5)	1 : 6000(5)	0
	37°C.	1 :70(5)	1 :100(5)	1 : 6000(4)	1 : 6000(5)	0
Tetrabothrius Adult	Rm.	0	0	0	0	1 : 6000(5)
	37°C.	0	0	0	0	1 : 6000(5)

difference was never greater than one dilution tube, which ordinarily is considered within the limit of experimental error.

Table IV was prepared from the data given in Tables II and III

TABLE IV

Relationship Expressed as Per Cent Values of Homologous Titers

Antiserum / Antigen	Homologous Titer	*Parorchis*	*Cryptocotyle*	*Siphodera*	*Plagitura*	*Zygocotyle*	*Moniezia*	*Dipylidium*	*Taenia taeniaformis*	*Tetrabothrius*
TREMATODA										
Echinostomatidae										
Parorchis	1 : 4000	100	12.5	7.5	1.67	0.25	—	—	0	—
	1 : 6000		8.33	5.0	1.12	0.17				
Heterophyidae										
Cryptocotyle	1 : 4000	12.5	100*	27.5	3.13	0	—	—	0	—
	1 : 6000	8.33		18.3	2.08					
Siphodera	1 : 4000	7.3	30.0	100*	0.75	0	0	—	—	—
	1 : 6000	4.87	20.0		0.5					
Plagiorchidae										
Plagitura	1 : 4000	1.67	4.38	1.25	100	0	0	—	0	—
	1 : 6000	1.12	2.92	0.83						
Paramphistomidae										
Zygocotyle	1 : 4000	0.33	1.12	0	0	100	—	—	—	0
	1 : 6000	0.22	0.75							
CESTODA										
Moniezia	1 : 4000	—	—	0	—	—	100	12.5	1.5	0
	1 : 6000							8.33	1.0	
Dipylidium	1 : 4000	—	—	—	—	—	10.0	100	2.8	0
	1 : 6000						6.6		1.87	
Taenia taeniaformis	1 : 4000	0	0	—	0	—	1.25	2.5	100	0
	1 : 6000						0.83	1.67		
Tetrabothrius	1 : 4000	—	—	—	0	0	0	0	0	100
	1 : 6000									

* Relationship not 100 per cent when 1 : 6000 is considered homologous titer.

and presents the percentage of relationship of the various species based on homologous titers of 1 : 4000 and 1 : 6000, which were considered to be 100 per cent. Since the titers of reactions between various stages

in the life cycle of a species were nearly the same, the percentage values in Table IV are the result of a consideration of all stages in the life cycle of the species under examination in determining the percentage relationship of that species with another.

Precipitin reactions of high titer were secured by injection of as little as 10 or 20 mg. of antigenic material, but the results were not consistent, since corresponding titers did not result in every case. Injection of 40 mg. of antigen apparently is necessary to obtain reliable results, though further work may reveal methods for securing consistent titers upon administration of much less antigenic material.

Noteworthy in the present experiments are the confirmatory results yielded by reciprocal tests, which would seem to be necessary if serological reactions are to be significant in determination or confirmation of phylogenetic relationships.

In Table V the alcohol-ether-soluble contents of the helminths have been tabulated in percentages of the total dry weights. The amounts of these materials varied from 2.7 per cent in cysticerci of *Taenia taeniaformis* to 41.3 per cent in adults of *Tetrabothrius*. That the lipid content of a single species is not constant may be concluded from the different values obtained in two different extractions of *Parorchis acanthus* or of *Moniezia expansa*.

TABLE V

Alcohol-ether-soluble Substances of Helminths
(In per cent values of total dry weights)

Species		
Parorchis acanthus (adult)	(*a*)	28.62
	(*b*)	32.82
P. acanthus (rediae)		25.30
P. acanthus (metacercariae)		14.88
Cryptocotyle lingua (adult)		29.76
C. lingua (cercariae)		20.35
Siphodera vinaledwardsii (adult)		25.00
Plagitura parva (adult)		4.95
Zygocotyle lunata (adult)		12.39
Z. lunata (metacercariae)		7.09
Moniezia expansa (adult)	(*a*)	25.00
	(*b*)	34.13
Dipylidium caninum (adult)		16.09
Tetrabothrius sp. (adult)		41.33
Taenia taeniaformis (adult)		17.53
T. taeniaformis (cysticerci)		2.73

(*a*) and (*b*) refer to two different extractions.

DISCUSSION

Among invertebrate Metazoa, research involving serological reactions has been confined largely to helminths. One difficulty in per-

forming experiments on helminths and other lower invertebrates is that extracts of tissues must be used and they apparently are not so simple, chemically, as blood sera. Another complicating factor is that investigators have used different techniques, both in making the extractions and in determining titers, and therefore the results are not strictly comparable. Certain investigators have used the dry weight of the powdered whole worm as a basis for determination of the titer, but since there is considerable variation in the proportion of original powder which can be brought into solution, it is better to determine titers from the weight of substances actually in solution. Others have based titers upon protein content of the extracts, but this method presumes that all the nitrogen is active immunologically and ignores polysaccharides, which are also antigenic. Thus, in the present experiments, nitrogen determinations of the filtrate were not made because it was felt that the polysaccharides in solution were also important for antigenicity and specificity, and information concerning the amount of *all* materials injected would give a better index of the specific titers expected than an approximate knowledge of the amount of protein, determined from its nitrogen content.

Protein constitution has been and still must be emphasized as the *basis* for morphological as well as physiological characteristics. Upon this fundamental and basic protein pattern, on which *species* specificity depends, polysaccharides or other substances may be superimposed to confer either an altered species specificity or an additional *type* specificity, such as the type specificity of certain bacterial polysaccharides. Campbell (1937) noted that, although whole worm antigens indicated specific identity for the ascarids, *Ascaris lumbricoides* and *A. suum*, their polysaccharides were immunologically distinct. These two ascarids, therefore, may be considered two varieties or strains of the same species.

While proteins, presumably because of graded chemical differences, provide gradual differentiation in serological reactions and phylogenetic relations, the lipids of closely related species, or even in different organs of the same species, may differ considerably, and the lipids of distantly related species may be very similar, if not identical, immunochemically. The reason for the non-specific influence of lipids is therefore obvious, and, since they are antigenic when in combination with proteins, their complete removal is necessary in tests designed to determine zoölogical relationships.

The positive reactions noted by Fairley (1919–1933), by Cawston (1921) and by Höppli (1921) in tests between the several species of schistosomes and between *Fasciola hepatica* and *Schistosoma* materials

are to be interpreted as cross or aspecific reactions resulting from the lipids present in the extracts which were used. Such group reactions may be of diagnostic value but are unsatisfactory for determining relationships.

Lipids were present in the aqueous extracts of helminths used by Canning (1929), and he observed a *tissue* specificity, i.e., different tissues of the same species were immunologically distinct. The more general reactions obtained in tissues, such as the sperm where the largest amount of nucleoplasm was involved, and the more specific in the egg, where the largest amount of cytoplasm was present. Since lipids in different parts or organs of the same species may be of diverse immunochemical composition and since lipids in combination with proteins are antigenic, it is possible that the *tissue* specificity reported by Canning is directly attributable to qualitative and quantitative variation in the lipid content of extracts of different tissues, even though, in certain reactions, the proteins may have been identical. In accordance with the two-fold system of species specificity proposed by Landsteiner (1928), the reaction titer of antisera produced by the injection of hapten-containing antigens would be the resultant of two types of specificity, specificity of protein and that of hapten. In Canning's experiments, since antisera produced by tissues with a low lipid content, such as sperm, yielded more generalized reactions within a single species, it is possible that the primary protein specificity was not entirely nullified or concealed by a lipid specificity. The anti-protein fraction of the anti-sperm serum would be predominant and react with the protein component of egg antigen. However, since egg and sperm materials are both derivatives of reproductive organs, there is reason to expect chemical similarity of their lipid components. Thus, the anti-lipid fraction of sperm anti-serum would react to some extent with the lipid present in egg antigen. The resultant titer, then, would express reaction of both anti-protein and anti-lipid fractions of sperm antiserum with protein and lipid components, respectively, of the egg antigen. Injection of antigens containing moderate to large amounts of lipid would alter the specificity of resultant antisera, from one dependent on proteins to one dependent on lipid. Consequently, since the quantity of lipid in muscle is much less than that in egg and since the lipids of the two tissues may differ qualitatively, precipitation of the anti-egg serum could not be produced by muscle antigen (low lipid content) if diluted to the titer of egg antigen (high lipid content). The observations involving materials of different species suggested to Canning that "the cytoplasmic materials, if modified in consequence of inter-species variations, show a radical change, whereas nuclear ma-

terials, if modified, are changed only in a few factors with many others remaining identical." Since lipids do not exhibit gradual phylogenetic gradations, it is possible that the lipid of *Ascaris lumbricoides*, which alters or overshadows the protein specificity, may differ so radically from that of the acanthocephalan, *Gigantorhynchus gigas* (= *Macracanthorhynchus hirudinaceus*), that no cross reaction occurred when egg materials were employed, though the proteins are closely enough related, chemically, to give cross reactions, such as those noted with sperm materials (low lipid content). On the other hand, lipoidal substances present in the antigens of distantly related species may have yielded group reactions, since the presence of a common haptenic lipid in two different species evokes production of precipitins which will react with either antigen. If cytoplasmic materials manifest greater inter-species variation than nuclear materials, the lipids in the cytoplasm may be the variable factors.

Previous to Canning's observations, the comparative value and importance of cytoplasmic and nuclear materials had been considered by Mez (1926), who stated that nuclear materials are the same in all parts of a plant and at all times during the life cycle. Moreover, he proposed that nuclear proteins *only* are serologically important in relationship studies. According to Boyden (1936), this proposal is "clearly wrong as it applies to animals, at least, for we have excellent cases of serological reactions indicating relationships based on egg albumins and serum proteins which cannot be due entirely to nuclear proteins." The proteins and polysaccharides of both nuclear and cytoplasmic materials and, indeed, of intercellular materials as well, are immunologically important. Consequently, it is probable that use of lipid-free antigens, whether nuclear or cytoplasmic, will yield *species*-specific and not *tissue*-specific antisera.

Specificity of antisera is dependent on the method of preparation of the antigenic solutions used to evoke antibody production. Antigenic material should be extracted at *low* temperatures or, if *room* temperatures are employed, the number of labile substances which could be altered during the aqueous extraction should be reduced by previous removal of the alcohol-ether-soluble components. In Eisenbrandt's experiments (1936, 1938), although buffered solutions were employed, many labile substances may have been lost or altered, since extractions were carried out on *fresh* tissues at *room* temperatures, and the lipids were not removed to effect a reduction in labile substances. The chemical nature of materials prepared in this manner might be so greatly altered that the resultant antisera would yield neither accurate or consistent relationships. An altered chemical nature of the antigens

prepared by Eisenbrandt was evidenced by the fact that different antigens produced antisera whose homologous reaction titers varied from 1 : 2000 to 1 : 2,048,000. In fact, homologous antigens were restricted by Eisenbrandt to "those actually used for production of antibodies and are not used in a general sense to mean the same species." This restriction would seem unnecessary if the extraction of a single species yielded similar antigenic solutions in every case. The qualitative and quantitative variation in lipids which may have been present, the different chemical alterations in the different extractions, and/or the antigenic capacities of the resultant different lipid-protein complexes of even a single species, may account for the great range in homologous titers.

In the experiments reported by Eisenbrandt, reciprocal tests agreed with the original ones in only a single case, and the failure of reciprocal tests, according to him, "complicated the matter of interpretation and prevented the data from being of primary importance in this work." Furthermore, he stated, "The indications are that little or no emphasis can be placed upon reciprocal data when saline extracts of helminths are used as antigens in studying phylogeny unless further fractionation of the antigens be employed." Illustrative of the widely divergent results obtained by him, *Toxocara canis* and *Dirofilaria immitis* exhibited a 0.001 per cent relationship one way and a 50 per cent relationship when tested reciprocally. Boyden (1926) had stated that "it would seem then that this principle of reciprocal relationships could be used as a test of the truth of the values obtained in the precipitin reaction. . . . Only those values which check within the limits of error of the reaction may then be taken."

Even if they were not responsible for non-specific precipitin reactions, lipids should be removed from extracts, because, as has been mentioned, spontaneous precipitates, attributed to lipids, were observed by Eisenbrandt (1938) in certain antigenic solutions and by Mackie and Anderson (1937) in normal sera.

When the amount of material injected is determined by protein content alone, and this protein content is low, the large amounts of haptenic lipids and antigenic polysaccharides administered along with the proteins may cause prozone (antibody excess) in the antisera, and low titers would result because reactions between antisera and highly diluted antigen are inhibited by the antibody excess, as noted by Hooker and Boyd (1935) and Marrack (1938). Aside from the proteins, the qualitative and quantitative composition of the antigens employed by Eisenbrandt is not known, but the results indicate that the amount of haptenic and other antigenic substances injected, in the

cases of extracts having a low nitrogen content, produced antibody excess, which was responsible for some relatively low titers of the homologous reactions. A constant amount (0.0096 gram) of nitrogen was injected, but, since the nitrogen content of extracts varied considerably, equivalent amounts of antigenic material were not administered. For example, almost three times as much *Ascaridia lineata* as *Toxocara canis* antigen would be injected to administer equivalent amounts of nitrogen. The resultant increase in non-protein *A. lineata* materials injected, most of which would probably be antigenic, might cause such an increase in antibody content of the serum that, as a result of antibody excess, an inhibition of precipitation would occur when the higher dilutions of antigen were employed in tests. Another possible explanation for the low titers observed by Eisenbrandt in cases of low protein content of the antigens is that the simple haptenic substances actually inhibit precipitation because they occupy the combining sites on the antibody molecules, thus preventing combination of true antigen and antibody, as has been reported by Marrack and Smith (1932) and Haurowitz and Breinl (1933).

In relationship studies, uniform, consistent results are dependent primarily on the technique employed in preparing the antigenic solutions and in performing the tests with antisera. In the present work, it is possible that some degradation of labile substances occurred, since the aqueous extraction was performed at room temperatures, but the preliminary removal of lipids eliminates at least a part of the materials which may be altered during a twenty-four-hour saline extraction. The lipid concentration of the worms varied from as low as 2.7 per cent to as high as 41.3 per cent, so the antigenic capacities of the extracts, incident to the lipids present, would have varied considerably if antigenic solutions containing lipids had been employed. Indeed, the quantitative and qualitative differences in the lipids of adult and larval stages of the same species might have yielded serologically distinct antigens, especially if these lipids were altered during the extraction. Not only the lipids present in the extracts, but also the temperature at which the precipitin reaction is performed, the time at which the end-point of the tests is determined, and the clarity of the antigen and antiserum are factors which could affect the titer. The titers of comparable reactions at two different temperatures in this series of experiments were usually the same, but the reactions were more definite at 37° C. As reported by Boyden (1926) and by Eisenbrandt (1938), a reaction time longer than one hour is undesirable because the layer of precipitate becomes diffuse and begins to settle out and the end-point would be less precise. Boyden also noted that, after filtration of the

antiserum, the titer usually increased because of the increased clarity of the serum and the consequent greater ease of detecting a fine ring in the last tube, but, if the antiserum or the antigen is cloudy enough to note such a difference in titer, the original determination should not be made on unfiltered material. By working with clear reactants at 37° C., titers may be accurately defined and species differentiated.

In accordance with the chemical maturation which occurs in vertebrates, it seemed that differences between adult and larval helminthic materials might be detected. Contrary to expected results, very little, if any, difference was noted in the titers of larval and adult materials used in the present experiments, so, apparently, larval proteins are as well-differentiated as those of the adult; at least, larval and adult antigens are not serologically distinct. Species specificity of reactions was maintained in tests with all stages in the various life cycles studied. This fact is important for life history studies, since larval stages may be identified with their adult forms by serological methods. Improvement in technique may yield satisfactory reactions upon the injection of less antigenic material, and, since the amount of material available is very limited in most cases, especially the larval stages of species which parasitize marine animals, such technical improvement would be advantageous.

In the present research, a species-specific titer of 1 : 4000 resulted from injection of aqueous solutions containing only 40 mg. of dried antigen from which the lipids had been removed. As noted, high-titered antisera were produced when only 10 mg. of antigen were administered, but the results were not entirely consistent. The relation between the amount of antigenic material injected and the homologous titers of resultant antisera has varied extensively in experiments reported by different investigators. In most cases, however, much more antigen was injected than in this series of experiments. The observations of Wolfe (1939), combined with the results of present work, make it seem very probable that the technique can be improved further to yield consistent reaction titers with antisera produced upon the injection of small quantities of antigenic materials. One should not decrease the amount of antigen injected, however, at the expense of a homologous titer below 1 : 4000, because the margin by which to differentiate species-specific from group-specific reactions would be too small.

"Species" of helminths may be defined tentatively as a group of organisms the lipid-free antigen of which, when diluted to 1 : 4000 or more, yields a positive precipitin test within one hour with a rabbit antiserum produced by injecting 40 mg. of dry-weight, lipid-free anti-

genic material and withdrawn ten to twelve days after the last of four intravenous injections administered every third day.

The species which were studied in this series of experiments were not allocated to their positions in the classification until the precipitin tests had been completed. Upon determination of family and subfamily affiliations, a surprisingly good correlation existed between the titer of the precipitin test and the degree of relationship of the worms as determined from their development and morphology. Of particular interest was the serological relationship shown by material of *Cryptocotyle lingua* and *Siphodera vinaledwardsii;* the positive reactions in such relatively high dilutions support Manter's (1934) allocation of *Siphodera* to the family Heterophyidae,[1] to which *Cryptocotyle* also belongs. *Parorchis acanthus*, belonging to the family Echinostomatidae, is more closely related to *C. lingua* and to *S. vinaledwardsii* of the family Heterophyidae than to *Plagitura parva*, a member of the family Plagiorchidae, or to *Zygocotyle lunata* of the family Paramphistomidae, since positive reactions in much higher dilutions were obtained with the heterophid materials. A closer relationship was expressed by the reactions between *C. lingua* and *P. parva* than by tests involving *C. lingua* and *Z. lunata*, which indicates that Heterophyidae are more closely related to the Plagiorchidae than to the Paramphistomidae. Furthermore, the kinship between *C. lingua* and *Z. lunata* was closer than that between *P. acanthus* and *Z. lunata*, indicating that the Paramphistomidae are more closely related to the Heterophyidae than to the Echinostomatidae. A heterologous titer of 1 : 600 between *Moniezia expansa* and *Dipylidium caninum* denotes a closer relationship between these two cestodes than between either species and *Taenia taeniaformis*.

It will be recalled that no cross reactions occurred between materials of trematodes and cestodes which were tested. The explanation for the negative results may be found in one of two possibilities or perhaps a combination of both. The amount of antigen injected (40 mg.) may have been insufficient to evoke antisera which would exhibit a *visible* reaction with antigens of distantly related species, though some combination of antigen and heterologous antibody may have occurred. On the other hand, the high salt concentrations resulting from the evaporation of the antigenic extracts to the desired dilutions may have inhibited precipitation or caused a delay in the reaction so no precipitate was present at the end of an hour. For certain antigenic

[1] Price (1940), *Proc. Helminth. Soc. Washington*, 7: 1–13, transferred the subfamily Siphoderinae Manter, 1934, from Heterophyidae to the related family Cryptogonimidae.

concentrations employed in the tests, solutions were evaporated to at least one-tenth of their original volume. Evaporation to this extent would change the salt concentration from 0.15 N to 1.5 N, which would be sufficient to cause a delay if not complete inhibition of precipitation (cf. Landsteiner and Welecki, 1911; Eagle, 1932). To bring some of the solutions to the desired concentration of antigen, the salt concentration would have increased well above 2 N. Had the salt concentrations not been so high, positive reactions between trematode and cestode materials might have resulted in certain of these experiments.

An objective test which reveals and expresses quantitatively not only differences in empirical chemical constitution but also slight stereochemical differences, which are the basis for distinction of closely related species, is available in the precipitin reaction, since the results which are obtained are independent of individual interpretations. Boyd (1937) correlated the cross-reactivity of various hemocyanins with their chemical similarity, and Landsteiner and van der Scheer (1940) concluded that cross reactions between proteins of kindred species are ascribable to similarity and gradual variation in determinant structures. In the present experiments, cross reactions with the lipid-free antigens indicate that true specific relationships depend on differentiation of species-specific proteins, but it is possible that modifications may result from the presence of polysaccharides.

Summary and Conclusions

The value of the precipitin reaction as a method for determining phylogenetic relationships of helminths has been demonstrated, since the reaction differentiated species and expressed relationships which are in agreement with those derived from morphological comparisons of the species studied.

Uniformity of results may be attributed to the following factors: vacuum desiccation of worms kept in the frozen state, preliminary removal of lipids from desiccated material, control of hydrogen ion concentration during extractions and tests, control of temperatures at which reactions were performed, and knowledge of the *actual* amount of material in the antigenic solutions. Positive reactions were more definite and the rates of precipitation were more rapid at 37° C. than at room temperatures.

Antisera produced by injection of lipid-free antigens yield species-specific serological reactions whereas those evoked by lipid-containing antigens afford no better than group-specific tests. Therefore, preliminary removal of lipids from the desiccated antigen is necessary to exclude the possibility of group reactions. Cross reactions with lipid-

free antigens indicate that true specific relationships depend on differentiation of *species*-specific proteins, but it is possible that modifications may result from the presence of *type*-specific polysaccharides, since purified, protein-free polysaccharides are precipitinogenic.

Consistent results were obtained by injection of aqueous solutions containing forty milligrams of dried antigen from which the lipids had been removed.

The titer of a reaction is the highest antigen dilution which gives a visible precipitate when tested with an antiserum, the amount of antigen being determined on the basis of dry weight of antigenic material *actually in solution*. Heterologous titers never exceeded homologous ones, and they required a longer time for expression. Titers of all reactions in these experiments were confirmed by reciprocal tests.

Except for two species, *Cryptocotyle lingua* and *Siphodera vinaledwardsii*, the reactions were species-specific in antigen dilutions of 1 : 6000; in view of these exceptions, a titer of 1 : 4000 must be assigned to species specificity.

Analysis of precipitin tests involving various stages in the life cycles of the trematodes, *C. lingua*, *Parorchis acanthus* and *Zygocotyle lunata*, and the cestode, *Taenia taeniaformis*, revealed that larval and adult antigens are not serologically distinct. Serological recapitulation does not occur since larval proteins are as well-differentiated as those of the adult. Accordingly, serological methods may be used to identify the larval stages of a parasite with the adult and thus aid in working life cycles. Furthermore, since chemical maturity is reached early, both larval and adult stages of helminths could be used indiscriminately in making antigens either for diagnosis of helminthiasis or for establishing zoölogical relationships by serological methods.

The precipitin reactions confirmed, in general, the classification and relationships of the helminths as determined from developmental and morphological considerations. The tests demonstrated that the trematode families Echinostomatidae and Heterophyidae are more closely related to each other than to the Plagiorchidae or the Paramphistomidae. A closer relationship was expressed by the reactions between Heterophyidae and Plagiorchidae than by tests involving the Heterophyidae and Paramphistomidae, and the kinship between Heterophyidae and Paramphistomidae was closer than that between Echinostomatidae and Paramphistomidae. The titer of reactions between the cestodes, *Moniezia expansa* and *Dipylidium caninum*, denotes a close relationship between these two species which is not equaled by the titers of either species with *Taenia taeniaformis*. No reactions occurred between the trematodes and cestodes which were tested.

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THE FUNCTIONAL ORGANIZATION OF THE NERVOUS SYSTEM OF ENTEROPNEUSTA

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Introduction

The chordate affinities of balanoglossids have been widely accepted ever since Bateson advanced the suggestion in 1885 and have not seriously been challenged since Spengel (see especially 1893). Some of the most characteristic features of chordates relate to the nervous system. Moreover, the evolution of that group has outstandingly involved this system. It is of considerable interest, therefore, to inquire into the nature of the nervous mechanism of the Enteropneusta. Investigations of a purely morphological type were made by the earliest workers, but we lack, as yet, any conception of the functional stage of development represented or the plan of organization. The purpose of the present paper is to report some simple experimental evidence concerning the physiologic pattern of the nervous system in the Enteropneusta, and to offer a tentative generalized picture of its condition.

The intimate relation between receptors, nervous mechanism, and effectors renders it appropriate, in the case of animals whose general behavior is unfamiliar, to consider all three systems as an integrated unit. Since our only knowledge of the activities of the first two named systems is gained through observations of the overt activities of the effectors, the latter system is treated first.

The original observations contained herein relate chiefly to two eastern Pacific species, *Saccoglossus pusillus* (Ritter) (formerly *Dolichoglossus*) and *Balanoglossus occidentalis* (as yet a manuscript species). These were collected near San Diego, California, and the majority of the experiments were carried out in the laboratories of the Scripps Institution of Oceanography. It is a pleasure to acknowledge the kindnesses extended to me by Dr. H. U. Sverdrup and Dr. M. C. Sargent of that Institution.

Effectors and the Character of Response

The behavior of enteropneusts originates in at least three effector systems: ciliary, glandular, and muscular.

Cilia

Balanoglossids exhibit an extraordinarily high level of development of the general ciliature, and of dependence thereon. The entire surface of the body, external and internal (with very limited local exceptions), is provided with cilia which beat, in all probability, essentially continuously throughout life. Such an elaborate and extensive mechanism might be expected to perform more than such localized functions as the creation of respiratory or nutritive currents.

Removed from their normal habitat and placed in an aquarium for observation, specimens of *Saccoglossus pusillus* employ their cilia as the principal locomotor organs! True, parts of the body move, muscularly, relative to other parts; and progression may be accomplished by a series of "inch-worm" like movements involving extension and fixation of the proboscis followed by contraction of the trunk. But this does not occur regularly (Benham, 1899, describes it in *S. otagoensis* but does not indicate how usual it is), nor does any snake-like pushing against minute obstructions in the substratum. Peristaltic progression may be observed, but, under these artificial conditions at least, slow progress of the whole animal by means of cilia represents the chief method of locomotion.

Cilia are also used in building sand tubes and in burrowing. They can be directly observed in the former process. In *S. pusillus*, as Ritter and Davis (1904) and I have observed it, the proboscis cilia play the dominant rôle. The proboscis exhibits continual exploratory movements, its tip touching the substratum now here, now there. Every sand grain adhering to the sticky mucus thereon immediately begins to move backwards over the surface of the organ. Thus develops a stream of rapidly moving particles which, however, stop their advance at the level of the collar; here they accumulate to form a short sheath. By the force of the continual stream of particles from the powerful proboscis cilia, the sheath is pushed back, eventually to cover the entire trunk. The spectacular nature and the speed of this process not only emphasize the importance (mentioned below) of a sand tube but also suggest the probable rôle of the cilia in burrowing. For, doubtless, these organelles importantly contribute in this process, especially in the Ptychoderidae and Harrimaniidae where the extensor muscles of the proboscis are even more poorly developed than in the Spengeliidae (van der Horst, 1927–39).

Other functions of the cilia are entirely conjectural. Since not even a plausible guess has been offered concerning the function of the gill slits in balanoglossids, the significance of the highly developed ciliature lining those passages remains unknown. Whether the cilia

are of importance in an exchange of fluids between the coelomic cavities and the ocean through the proboscis and collar pores remains, with the antecedent question of the existence of such exchange, unproven. Notable as the only experimental evidence on the question is Bateson's (1885) demonstration that particles injected into the proboscis or collar coelom are ejected through the respective pores; but he found no intake of water, such as has frequently been thought to dilate the proboscis in the cycle of digging movements (e.g. Spengel, 1893).

Are the cilia of balanoglossids under nervous control? No work has been specifically directed at this point, but some observations are suggestive. The cilia beat, as would be expected, in the absence of nervous function, thus in anaesthetized animals and in the embryo before a nervous system has appeared. That is, ciliary activity is not dependent on the nervous system, a property generally ascribed to all cilia. The question which remains is: Can the nervous system modify the activity of the cilia? Since Parker's dictum in 1919, ". . . in fact, it may be stated that at present there is not the least ground for the assumption that true nervous activity is in any direct way involved in ordinary ciliary reactions," [1] there has been brought forward convincing evidence of nervous modification of ciliary activity in several cases (Göthlin, 1920; Alverdes, 1922; Copeland, 1922; Merton, 1923; Carter, 1926; McDonald, Leisure and Lenneman, 1928; Seo, 1931). It would seem from the evidence available that, over and above the intrinsic, epithelio-genic beat and coördination of all ciliary fields, some ciliary fields have come under nervous control. In such epithelia the normally continuous beat may be stopped, started, reversed or otherwise modified over the whole or only part of the field, at the "will" of the nervous system. Carter (1926) has set forth a rigid series of specifications for the thoroughly satisfactory demonstration of a case of nervously regulated cilia. No one has sought to satisfy these criteria for the balanoglossids, but, doubtless, many have observed, as I have, the striking behavior of the epithelium of adult enteropneusts in life. Here is seen an impressive degree of ciliary reversal and inhibition. Not whole fields but very small areas stop beating and start again many times in a few seconds, or only occasionally. Localized changes in direction of beat likewise occur. Now considerable, now limited areas of epithelium beat, now dorsally, now obliquely posteroventrally, dorsally again, anteriorly, anteroventrally, ventrally, shifting in rapid succession, the environment meanwhile remaining to the human senses uniform. Such remarkable behavior is difficult to explain,

[1] The qualification "ordinary" is puzzling. Nowhere in Parker's subsequent discussion does he refer to any extraordinary ciliary activity that might form an exception to his generalization.

in the absence of external stimuli, on the basis of an intrinsic epithelial mechanism acting entirely independently, but, of course, additional evidence is desirable before a nervous control is assumed. These features of ciliary activity were observed in *Saccoglossus pusillus* and only on the surface of the trunk, the proboscis cilia beating steadily posteriad.

It would seem probable, then, that the unusually extensive and elaborate ciliary mechanism of balanoglossids is of profound importance to the economy of the animal, at least in certain kinds of locomotion, in sand tube formation and burrowing. The occurrence of specialized local ciliary apparatus and the complex behavior of certain ciliary fields indicate that it is probably important in other more specific and complex functions. The occurrence, speed, and localization of inhibition and resumption as well as reversal of ciliary beat suggest that the present animals may offer a new case of nervous control of cilia.

Glandular Activity

Enteropneusts are among the most copious producers of mucus known. Furthermore, to reason from the variety of histologically differentiated types of unicellular epithelial glands, they must elaborate a considerable array of different substances. The nature, importance, and modifications in secretion of these substances remain virtually unknown. From the highly developed state of the mechanism, the volume of its production and the habit of the animals, it seems safe to regard this system as of great importance. But no evidence can be cited bearing on the question of control of glandular activity beyond the facts that anaesthetized worms and isolated strips of epithelium continue to secrete. Probably largely independent of the nervous system, the existence or nature of direct or indirect nervous control cannot as yet be surmised.

Light Production

Several species of balanoglossids have been reported to produce light upon stimulation after dark-adaptation (Kuwano, 1902; Crozier, 1917). The nature of the phenomenon has not been determined in these animals. Crozier assumes that it is intrinsic, i.e., that the light is produced by the worm itself, and it may be that some of the plethora of unicellular integumentary glands are responsible. In this case, of course, the phosphorescent organs should not be listed as a distinct effector system. The only facts known about the phenomenon are that it is elicited by external stimuli such as poking the worm or tapping the aquarium, that it is inhibited by exposure of the subject to light,

and that it is accomplished as well by small fragments as by intact animals. Regarding the question of nervous control, Crozier argues from the last-named fact that light production must be independent of the nervous system. But, in view of the high degree of neuromuscular autonomy of even small fragments, demonstrated later in this paper, his position is no longer tenable, and the first two facts named above suggest reflexes involving the nervous system.

Muscular Activity

The normal habit of life of all enteropneusts is doubtless benthonic. Many species are burrowers. Their food habits though not yet elucidated are clearly passive, for in general these animals are sand-eaters and obtain their nourishment from organic matter contained in the substratum. Their means of protection, as well, seem likely to prove passive, though even less understood than the means of nutrition. Very possibly the well-known iodoform odor of many species functions in this connection and perhaps we may thus account for some of the diverse integumentary glands.

Corresponding to such a passive mode of life, balanoglossids are among the most sluggish of all free-living animals. Not only are their movements characteristically sluggish but the observer is struck by the small number of different responses possible. The movements incident to burrowing and to advance and retreat within the burrows, comprise the sum total of muscular exertions of most of these animals.

Cases of an extraordinary degree of activity for enteropneusts are the reports of Ikeda (1908) and Spengel (1909) of the pelagic occurrence of two species of the genus *Glandiceps*. Swarms of individuals were encountered swimming at the surface under conditions which could not be correlated with breeding phenomena or any other obvious factor. The mechanism of swimming is not made clear.

In view of the frequent misconceptions thereof, a word may be said concerning the mechanism of burrowing. Most importantly, it is not yet clearly understood, even in essential respects. The use of the coelomic pores for dilation and collapse of the proboscis and collar with sea water has not been demonstrated and is, on several anatomical grounds, unlikely. The service of the "notochord" and its secreted skeletal sheath is principally that of the muscle attachment, secondly that of strengthening the proboscis-collar junction; but it is not to be thought of as providing an otherwise flabby proboscis with the rigidity required for digging in heavy mud and sand. For that organ, in short- as well as in long-proboscis species, *is* flabby, and balanoglossids burrow slowly and inefficiently because of their lack of rigidity. The muscles principally responsible for forcing the proboscis into the sub-

stratum, i.e., the extensors of that organ, are the circular muscles which are conspicuously poorly developed in all species, and especially so in the Harrimaniidae and Ptychoderidae. Much more voluminous are the longitudinal muscles which shorten and, incidentally, swell the proboscis. (The disproportion is even greater in collar and trunk.) These facts emphasize the probabilities that: (1) those balanoglossids called, above, "burrowers" are chiefly livers *in* (more or less permanent) burrows, not active diggers, and (2) cilia are very likely of great importance in burrowing, as in sand-tube formation (very likely in the same way) and in locomotion.

To emphasize the paucity of clearly-defined and diversified movements, the results of the search for responses lending themselves to physiologic testing in the experiments described below may be cited. Using *Saccoglossus pusillus*, I was able to find but a single reflex even approaching an adequately dependable and clean-cut piece of behavior for which to test experimental subjects. This is elicited by tactile stimulation, for example, a gentle poke with a probe, applied to the proboscis. If each of a series of conditions having to do with the state of the specimen has been satisfied, such a stimulus results in the most rapid muscular response this animal is capable of, namely, a contraction of the longitudinal muscles of the trunk. The trunk is thereby shortened and coiled, but the collar and proboscis need not be involved in the movement at all.

The predictability of this "shortening reflex" depends most on the following conditions. The specimen must be in good condition, uninjured in collection except as regards the abdomen, which may even be missing without affecting the response. It must have been but recently removed from its burrow, whether in mud flat or aquarium. It should be active, moving about the vessel in exploratory fashion; the best responses are obtained from animals stretched out straight, in forward, doubtless ciliary, progression. And, finally, it must not have executed a generalized contraction within the preceding five minutes.

This is, in all probability, a true nervous response, since it takes place at a distance from the point of stimulation and is abolished, reversibly, by light anaesthesia such as does not impair ciliary or glandular activity or irritability of the muscles to strong direct stimulation.

We have here to do with a motor apparatus consisting of smooth fibers, not segregated into discrete muscles but occurring in loose masses or layers. It is functionally characterized by sluggish action, generalized responses, a lack of differentiated reflexes, and a relatively simple rôle in a rather passive, benthonic habit of life.

Receptors and the Known Adequate Stimuli

Although several authors have suggested a sensory nature for certain peculiar structures of the integument (Spengel, 1893; Assheton, 1908; van der Horst, 1930; Brambell and Cole, 1939), the lack of adequate reason for so regarding them makes it necessary to say that as yet no sense *organ* has been demonstrated in the Enteropneusta. This is a very astonishing fact, considering the early origin of differentiated organs of reception, and indicates not only a very simple type of receptor mechanism, but the probability of a very generalized type of irritability. The receptors are assumed to be scattered sense cells in the epithelium. Only one author has claimed to have seen sensory cells in balanoglossids, Hess (1938) having identified certain large basiepithelial, bipolar cells as photoreceptors.

The facts known about sensory physiology in enteropneusts fall into three groups according to the nature of the stimuli: mechanical, chemical, and photic.

Mechanical Stimuli

The irritability to probing and to tapping of the aquarium has already been mentioned. Characteristic responses, varying with the species, result from the stimulus of contact with a sandy or muddy substratum.[2] Species-characteristic behavior in response to exposure by the tide is also reported. This involves retraction or protrusion of the proboscis, or migration up or down in the burrow (Ritter and Davis, 1904; Morgan, 1894; Assheton, 1908; Stiasny, 1910). Disturbance of the substratum, as by footfalls, brings about quick retraction in the burrow.

Chemical Stimuli

We know virtually nothing concerning the sensitivity of balanoglossids to dissolved chemicals. The single report on record is the statement of Crozier (1915) that "for the chlorides of the alkaline metals, the normal lyotropic series, $K > NH_4 > Li > Na$, was found to express their stimulating efficiency; this was mainly a kation stimulation, but in the case of salts ($CaCl_2$, e.g.) which did not stimulate strongly, other anions (in this case $Ca(NO_3)_2$) were effective as stimulating agents." Crozier, further, was able to separate, by exhaustion or anaesthesia, the mechanical-chemical irritability from photic sensi-

[2] The curious response of *Saccoglossus pusillus* to the absence of mud is death, within as short a time as two or three days. Whether this is due to starvation, exhaustion or some other factor has not been determined, but the suddenness of death and the rapidity of cytolytic disintegration even in well-aerated, darkened aquaria are spectacular.

tivity, but the two former types of irritability could not be physiologically separated. He concluded that there are generalized receptors open to both mechanical and chemical stimulation, but recognized that these facts are "insufficient proof of the separateness of the sensory organs concerned in the reception of photic stimulation." He felt, however, that they do show "that photic irritability depends upon a process distinct from that implicated in the other modes of stimulation."

Photic Stimulation

Crozier noted in 1915 that *Ptychodera* and *Glossobalanus* showed "differential sensitivity" to light, and were indeed negatively photokinetic. Pursuing this form of experimentation, he found (1915, 1917) that: (1) The sensitivity to light was enhanced by dark-adaptation. (2) Photic and other forms of irritability could be readily separated physiologically (see above). (3) The collar nerve cord is not necessary for the kinetic response to light. (4) Posterior fragments or pieces of abdomen, including the caudal tip, are able to respond, but move away from the source of light tail first! (5) The tip of the proboscis is most sensitive, although the whole body exhibits some irritability. (6) Exposure to light greatly inhibits the ability of these animals to produce light, though such production could always be brought about by moderate induction shocks. (7) Even small fragments are able to produce light and are inhibited therefrom by exposure to light.

The only other experimental work upon enteropneusts has been that of Hess, whose interest was primarily in the photic response of these animals. Hess (1931, 1936, 1937, 1938) confirmed the observations of Crozier and then attempted to analyze the response by removing various regions of the body. He reported that the response (orientation and movement away from the source of light) was unimpaired by cutting off the proboscis, or the dorsal third of the collar (to remove the collar nerve cord), or its ventral third (to remove the "ventral nerve cord" of the collar [a cord which does not exist]), or the trunk and proboscis (leaving only collar and "peduncle" or proboscis stalk). The isolated proboscis, likewise, was able to respond. But, pieces from which the proboscis and its peduncle had been removed (or proboscis, peduncle, and collar, i.e., isolated trunk fragments) were unable to orient with respect to light. He concludes that the peduncle contains a "nervous center" concerned with light reactions, a center of which the proboscis, unlike the rest of the body, is independent. In *Ptychodera bahamensis* Hess considered the reactions sufficiently clean-cut to take reaction times. For all regions or combinations except the isolated proboscis these times were very nearly the same (1.90″–2.77″),

including the, presumably, optimal combinations—peduncle, collar, and trunk—as well as abdominal fragments which cannot orient but only, it is to be inferred, squirm aimlessly. The reaction time of the proboscis is given as 25.23″. This remarkably slow result Hess explains as being due to the fact that the proboscis has now been severed from the "central nervous system." That this "most sensitive region of the body," which can, when isolated, orient and move away from light, should lack this nervous ability—a rapid light response—which is possessed by a trunk fragment, is difficult to believe. His interpretation (or the figure for the reaction time of the proboscis) seems the more dubious (1) in view of the concept, arrived at below, of the nature of the "central nervous system," (2) in view of the independence of the light response of the great nervous concentrations in any case (Crozier, 1917, and below), and (3) in view of the general behavior and properties of the proboscis.

In 1938 Hess repeated on *Saccoglossus kowalevskii* those experiments involving intact animals. Again, intensities of light above a certain minimum resulted in negative orientation with little tendency to trial and error movements; but weak illumination produced a tendency to orient toward the source. Pin-point light exploration showed all regions to be sensitive, but some regions more so than others, the proboscis being the most sensitive.

My own experiments with photic stimulation were performed on *S. pusillus*. This species was found to be definitely negatively tropic although its response was very soon lost after removal of the animals from mud and exposure to light. The photic response is abolished by light anaesthesia, but returns after brief exposure to clean sea water. This shows, following Parker (1919), that the reaction depends upon the nervous system.

The methods of investigation used by Hess on *Ptychodera* presuppose an extended, straight animal, relatively motionless before exposure to light, capable of turning promptly upon exposure in a definite direction and without trial and error or "indecision." *S. pusillus* rarely assumes any but a coiled position with only the proboscis relatively straight, almost continuously moves about in exploratory fashion if fresh and healthy, and responds, often (especially in specimens removed from mud for some time, as in operatives and their controls which are rested in a constant temperature darkroom for at least one hour) with considerable hesitation. These differences render inapplicable to *S. pusillus* the technics of Hess, especially the determination of reaction times. But the existence of photic irritability was clear and the regional assignment of responsibility for the negative orientation to light was quite possible.

Hess's (1937) method of analysis was repeated upon variously constituted fragments of the body. After operation these fragments were dark-adapted at 20° C. for 1 to 2 hours and then exposed to a laterally incident beam of light with the following results. (1) Isolated proboscides, cut well forward of the peduncle, can regularly turn away from the source of light. (2) As would be expected, fragments consisting of proboscis and peduncle or of proboscis, peduncle, and collar readily respond and orient themselves in the same way. (3) Specimens with the collar mutilated, either by cutting across or removing the entire dorsal third (to include the collar nerve cord) are perfectly able to orient with respect to light. Controls with the ventral third of the collar missing or transected respond similarly. (4) Fragments of trunk region are incapable of orientation with respect to the direction of illumination. (5) Pieces consisting of collar and trunk but without peduncle or proboscis are incapable of orientation. Thus far all results correspond exactly with those of Hess. But (6) pieces similar to the last-named with the addition of the peduncle, i.e., with nothing removed but the proboscis, never succeeded in turning away from the light source in these experiments. The operations in this case were carefully performed so as to leave intact the entire peduncle and base of the proboscis, since this result is diametrically opposed to that reported by Hess for *Ptychodera*. It was found, by depriving further specimens of *Saccoglossus* of various fractions of the proboscis, that nearly one-third of its length must remain attached to the peduncle to obtain occasional definite responses, and more than one-third for consistent negative phototropic movements. It must be recalled that *Saccoglossus* is characterized by an extremely long proboscis; when two-thirds of it has been removed the remainder still exceeds the collar in length and is, proportionally, longer than the intact proboscis of *Ptychodera*. The long proboscis permits that much more exact delimitation and transection. The present conclusions, therefore, may be stated thus: although all regions of the body are sensitive to light, responding by aimless squirming, only the proboscis is capable of orienting with respect to the source of light, and the normal response of the intact animal is dependent upon that organ. The assumptions of a "center" in the usual neurologic sense, or of a central nervous system are not justified by the evidence at hand. Hess's results are open to the same interpretation, if it be supposed that his operation corresponding to (6), above, left a minimal amount of the (very short) proboscis still attached to its peduncle to orient the preparation to light.

The general nature of the sensory physiology of balanoglossids is,

therefore, clear, though the details are only sketchily indicated. Generalized receptor cells are scattered throughout the integument though differentially concentrated. The proboscis is most sensitive and, in a general way, a gradient of decreasing sensitivity from anterior to posterior exists. The senses of enteropneusts are at a very low level of development and it would seem probable that the animals are not greatly dependent on them. This condition may be correlated with the essentially passive, bottom-living habit of life. Though sensitive to a variety of forms of stimulation, the few and generalized

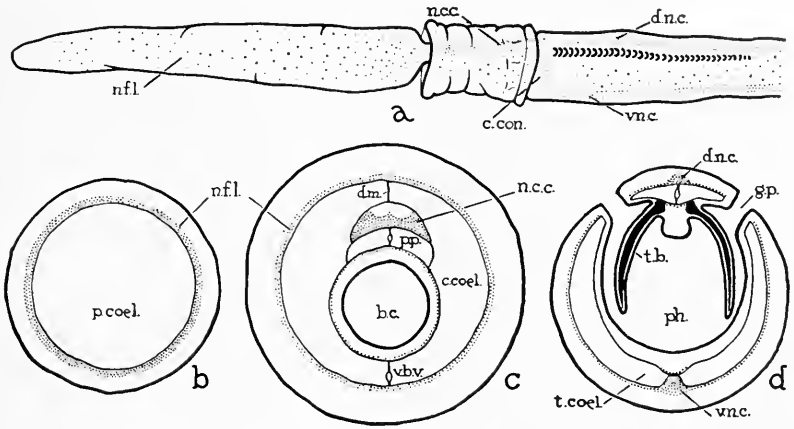

FIG. 1. Schematic representation of the nervous system of *Saccoglossus pusillus*. The nerve fiber layer of the epithelial nervous system is indicated by stippling. (*a*) Lateral view of proboscis, collar, and anterior end of trunk. (*b*) Transverse section of proboscis, (*c*) of collar, (*d*) of pharyngeal region of trunk. Organs occupying coelom have been omitted. *b.c.*, buccal cavity; *c.coel.*, collar coelom; *c.con.*, circular nerve connective; *d.m.*, dorsal mesentery; *d.n.c.*, dorsal nerve cord of trunk; *g.p.*, gill pore; *n.c.c.*, internal nerve cord of collar; *n.f.l.*, nerve fiber layer of superficial plexus; *p.coel.*, proboscis coelom; *ph.*, pharynx; *p.p.*, perihaemal pocket of trunk coelom; *t.b.*, tongue bar (coelom not shown); *t.coel.*, trunk coelom; *v.b.v.*, ventral blood vessel; *v.n.c.*, ventral nerve cord of trunk.

kinds of responses suggest a low order of differential recognition of stimuli, and little or no differentiation of "specific nerve energies." The beginnings of complexity are seen, for instance, in the dependence of the whole upon one part for orientation to light, but on the whole the level that has been achieved in this group is lower than that of many of the coelenterates—the simplest animals having any nervous system.

Functional Organization of the Nervous System

Anatomically, the nervous system of balanoglossids, as described by its discoverer (Spengel, 1877, 1893, and not since significantly modified), consists (Fig. 1) of a general superficial plexus located at the

base of the outer epithelium everywhere over the body and locally thickened to form so-called cords in the mid-dorsal and mid-ventral lines of the trunk—through the dorsal coelom of the collar as the only internal part, and again on the surface of the peduncle. All of these regions of nervous concentration, except the collar cord, are continuous with and connected by the general plexus; thus the peduncular thickening and the proboscis plexus, the dorsal and ventral trunk cords and the lateral trunk plexus are continuous basiepithelial layers. The ventral cord is confined to the trunk and at its anterior end a circular connective, traversing the collar-trunk boundary, passes from it to the dorsal cord where that is continued into the collar coelom.

The most important feature of the neuromuscular organization of these animals, and one profoundly conditioning the character and possibilities of experiment on it, has already been emphasized, namely, the simplicity and lack of variety of responses. Not only this, which could be said of an animal with a single, clean-cut, invariable response, but the responses of *Saccoglossus pusillus* are not uniform and dependable. The single sufficiently predictable reflex found in this species has been described above as the "shortening reflex."

The Nerve Cords

If the dorsal nerve cord of the trunk in a healthy specimen of *S. pusillus* be transected a little behind the collar (the technic of the operation and the extent of the cut make virtually no difference; a single stroke with sharp iridectomy scissors was found most convenient), a preparation is obtained in which the shortening reflex is hardly impaired. Often it is less certain, sometimes slower, but many responses are obtained which cannot be distinguished from those of normal subjects. Using *Balanoglossus occidentalis*, a large species only occasionally obtainable, this result is even clearer. It must be said that all experiments with this species were performed upon larger or smaller fragments, since I have never seen an intact specimen. However, it is easily demonstrable with other species, and has indeed been well known to earlier workers, that loss of the posterior part of the trunk has no noticeable effect on behavior. With this species it is possible to show, not only a nearly normal posterior response to anterior stimulation, but also a proboscis response to trunk stimulation virtually unaffected by transection of the dorsal nerve cord.

Similar operations affecting the ventral cord, however, have a marked effect on the shortening reflex. It is greatly impaired posterior to the level of the cut, is slower, prolonged as sluggish writhings, and has a higher threshold of stimulation. Again, the results with

Balanoglossus are even more clean cut. The animal ceases its spontaneous exploratory movements, but spontaneous peristaltic waves pass back from the level of the cut, as in isolated trunk fragments.

The effect of transection of both cords is even more pronounced. Now the response is virtually abolished posterior to the level of the operation. Stimulation adequate for the controls is entirely ineffective. Stronger prodding of the proboscis may elicit contraction of proboscis, collar, and trunk muscles anterior to the cuts, and a few of the contiguous muscles immediately posterior thereto. Repeated, relatively strong stimulation does, however, invariably produce movements of the abdomen, although not of the same character as the normal rapid shortening.

Lateral incisions, in the frontal plane, at the junction of trunk and collar, one on each side, yield results similar to the ventral cord transection above. Such incisions interrupt the continuity of circular connectives which thus appear to be of considerable importance in normal communication between the proboscis and the ventral cord of the trunk; that is, the alternative path through the general lateral plexus is not the route normally employed in rapid long distance reflexes. If the dorsal cord of such an individual be severed, the properties of the resulting preparation are precisely those described in the last paragraph.

Transverse cuts through the dorsal third of the collar will include the collar nerve cord. Such an operation results in animals markedly less active than normal, resembling in this feature and in the greatly impaired shortening reflex animals with both trunk cords interrupted.

It is curiously difficult to observe a ganglionic or modifying function in the great cord of the collar which, from the anatomical evidence, has been thought to be a central organ. I have failed as yet to devise an experiment to demonstrate such a function. Its strategic and unusual position, along the path of the main reflexes, crucially affects its accessibility to experimental analysis. The great nerve centers of other animals are usually terminal, but here we are unable to isolate the ganglionic function without interrupting the main nerve paths that pass through the collar cord. It is perhaps significant that nothing more can at present be said than this: No effect of cutting the collar cord has revealed itself except such as can be explained as a parting of pathways. Thus burrowing and photic reactions are quite normal after collar cord section (or removal); but the shortening response of the trunk to proboscis stimulation is impaired in the same way that it is after interrupting the cords of the trunk.

To test the effect of the operations *per se*, aside from the interrup-

tion of nerve cords, I have made various mutilations of the body wall in regions not traversed by concentrations of nerve tissue. These have no detectable effect on the ability to jerk up the abdomen upon gentle proboscis stimulation. However, more drastic operations, such as removal of the anterior third or half of the proboscis, have a noticeable effect, at least temporarily. Such subjects show a decrease in spontaneous movement, especially exploratory progression, and are rarely stretched out straight, but remain coiled and quiet. These characteristics are relative; no profound change is found in the subjects in question. They show no loss in ability to respond normally to proboscis stimulation.

Section of the cords in isolated fragments of trunk region reveals a further detail of their function. Whereas the branchio-genital and

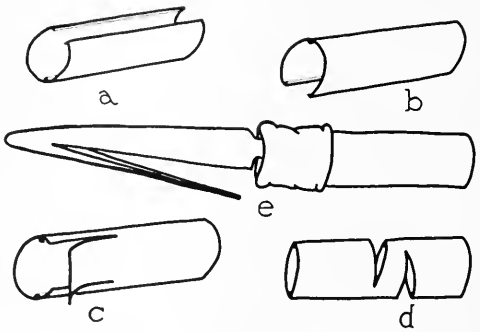

FIG. 2. Diagrams of operations on fragments of balanoglossids. See text for explanation.

especially the abdominal regions of intact animals exhibit almost continuous writhing movements, a length of trunk cut off from collar and proboscis lies motionless for many seconds or longer. Moreover, the occasional waves of contraction which do initiate in such pieces (especially branchial and terminal regions) or the slow peristaltic waves elicited by external stimuli are interrupted by interruption of the cords. If both cords are cut, only a few muscles immediately behind that level contract. If only one cord is cut, the waves are stopped, but not as sharply. Occasionally such a contraction wave starts up again after a delay of one half second or more and progresses to the level of the next cut. The ventral cord appears to be more influential, the dorsal somewhat less important, although the evidence for this difference is not here as clear as above.

A curious difference between dorsal and ventral nerve cords is revealed by the following experiment. This, further, anticipates the

next section by demonstrating that impulses are conducted through the lateral plexus, in this case between the cords in the transverse plane. A length of trunk region is opened by a mid-dorsal longitudinal slit (Fig. 2, *a*) and stimulated anywhere along the cut margin of one side. Moderate stimulation suffices to elicit not only a muscular response on the same side but also contralaterally. An even weaker stimulus may produce an ipsilateral contraction alone. The reciprocal preparation, however, opened by a mid-ventral slit (Fig. 2, *b*), will not, even upon strong stimulation, produce a contralateral response, although contractions of the same side are easily aroused. These facts indicate some difference in the pattern of the nerve cords such that impulses readily cross the ventral but not the dorsal cord. The ability to pass across the midline would seem, *a priori*, to be primitive; on this assumption the dorsal cord is more specialized than the ventral, although, as indicated by the previous experiments, the latter is the more important.

Such are the effects manifested in the first few hours after operating. What, now, happens to such operatives when kept, undisturbed, under favorable conditions, for some days? Of a group of twelve specimens so treated, nine recovered normal ability to respond within ten days, four of these being normal by the fifth day. Of the remaining three, two died and one recovered its active behavior but not its normal, rapid response. Among the animals which recovered completely were subjects which had suffered transection of the collar cord and others whose dorsal and ventral nerve cords had been interrupted in the trunk. Complete tissue regeneration had not occurred, but, histologically, fibrous continuity of the cords was reestablished to a degree which was evidently sufficient to reestablish functional paths even in these short periods.

Experiments on the General Plexus

It has already been indicated that the body wall aside from the nerve cords is able to conduct nervous impulses. This, it seems logical to assume, probably takes place through that thin basiepithelial nerve fiber layer which has been referred to as the general plexus. The present section is devoted to an elucidation of its properties.

Diffuse Transmission.—The experiment with trunk segments slit open longitudinally demonstrated dorso-ventral conduction (actually involving an arc in the transverse plane). By stimulating first one side and then the other of the first-described preparation, it can be seen that the lateral plexus is conducting now dorsally, now ventrally. The threshold of stimulation for conduction in this direction is, furthermore, low relative to that in the next several experiments.

It was noted above that, after section of the great conduction tracts (collar cord or both trunk cords), persistent, strong stimulation of the proboscis will elicit contraction of the abdomen, although this contraction is delayed and abnormal in character. This suggests the existence of alternative, though poorer and slower paths. The general plexus can, therefore, conduct anteroposteriorly. In *Balanoglossus occidentalis* it was possible to demonstrate conduction anteriorly as well as posteriorly.

Such behavior is highly suggestive of a nerve net, for the property of diffuse transmission is characteristic of such nervous systems and is not found in others. The anatomical relations, as far as known, correspond with those of typical nerve net systems but the distinguishing features of the nerve net are more important physiologically than anatomically; moreover, the functional characteristics are more easily and certainly ascertained than the crucial histologic relations.

As illustrative of a number of other experiments demonstrating conduction through the lateral plexus, the following may be described. A long piece of trunk region is incised longitudinally just lateral to the dorsal cord and again just to one side—the same side—of the ventral cord, the incisions extending through about half of the length of the piece. There is thus produced a preparation (Fig. 2, *c*) from which projects a tongue of lateral body wall with no nerve cord, still attached, however, to the remainder of the trunk in which the cords are intact. The cordless tongue being now strongly stimulated, a delayed bilateral response in the uncut posterior end follows. This is much clearer in *B. occidentalis* than in *S. pusillus*, doubtless because the former's larger size permits a neater operation. In this species the response even extends forward into the unstimulated anterior region. Stimulation of this region, provided as it is with intact cords, produces prompt contraction of the posterior end, and need be only a fraction of the strength or duration of the stimulus required in the case of the cordless tongue. If the cords then be severed at the base of the tongue, the anterior region still possessed of cords will behave precisely as did the cordless tongue in respect to higher threshold and delayed conduction.

Again, a trunk fragment may be deeply notched, twice, transversely, one incision extending from the dorsal side through at least two-thirds of the animal's diameter, the other from the ventral side and about 5 mm. in front of or behind the first (Fig. 2, *d*). Strong stimulation anterior to the operations will elicit posterior responses, indicating the conduction of impulses around the overlapping obstacles, but with considerable difficulty. Occasional spontaneous contractions of these pieces were noticed. Beginning near one end

these spread as waves, apparently unimpeded by the cuts, to involve the whole fragment in contraction at the same time.

In all of these and other similar experiments it was noticed that much greater difficulty is encountered in anteroposterior transmission than in dorso-ventral. Much stronger stimuli are required for the former than for the latter.

The proboscis is especially favorable for demonstrations of diffuse transmission by reason of the absence of differentiated conduction paths. The proboscis may be slit nearly in two in any direction and yet respond as a whole to adequate stimulation of either half. This statement must be qualified since the proboscis does not respond to stimuli in the simple, certain manner of other parts, although it is the most sensitive region. It is as likely to continue its aimless motions as to react clearly to a prodding which suffices for the jerking up of the abdomen. The most dependable form of this experiment takes advantage of the fact that response of the posterior regions to proboscis stimulation is more reliable than its own response. The proboscis is cut obliquely, from the posterior margin toward a point on its main axis near the anterior end and in any plane about that axis (Fig. 2, *e*). The stimulus is then applied to the posterior end of the free tongue. The impulse is propagated anteriorly, around the cut, and then posteriorly to elicit a trunk shortening. Or the same experiment can be executed on preparations with the proboscis transversely and deeply notched, from any side, showing the absence of special conduction paths and the ability of the plexus to conduct impulses over whatever fraction of itself remains intact.

But the simplest demonstration that transmission may be truly diffuse utilizes fragments of isolated proboscis, thus eliminating any possible central nervous influence. Such fragments may be stimulated at any given point by a single contact stimulus and the response observed to take place all around this point and to involve a rapidly widening circle, i.e., impulses are being conducted in all directions away from this spot. Another stimulus at a neighboring point elicits the same behavior. But now some of the same paths are being traversed in the opposite direction. An infinity of different points may thus be stimulated with the same result and impulses shown to pass in both directions between any two of them.

Conduction with Decrement.—It is especially easy with the proboscis of *B. occidentalis* to demonstrate that the response to a stimulus spreads in every direction from the point of stimulation but only to a certain distance. Furthermore, this distance varies according to the strength of the stimulus, so that a larger area is called into muscular

contraction by a stronger stimulus. Again, we find a property characteristic of typical nerve nets (Parker, 1919) manifested in balanoglossids.

Autonomy.—The parts of the body of balanoglossids are remarkable for the degree of autonomy they exhibit. This property is highly significant from the point of view of the organization of the nervous system and we may describe its manifestations in isolated fragments of these animals.

Isolated proboscides immediately strike the attention by reason of their activity and irritability. They live well for many days. (I have never had opportunity to keep fragments long enough for any considerable amount of regeneration.) They respond to touch and to light as well as in the intact worm, move about in an exploratory manner—by means of cilia, and contract vigorously. But they are not as successful as are whole animals at burrowing, possibly for lack of weight and length in obtaining a purchase on the substratum. The most clearly autonomous major region, the proboscis, behaves alike attached or unattached to collar and trunk.

The isolated collar fragments which I have observed lived but one or two days before disintegrating. They were capable, however, of responding locally to touch and light. This region performs few muscular movements itself either in the intact animal or when isolated and most of its organs are of significance chiefly for the whole organism (collar cord, great blood vessels, buccal apparatus, specialized integumentary glands, skeleton). It has, nevertheless, the neuromuscular equipment to respond autonomously to stimuli.

Isolated trunks or parts thereof seem quite normal. Characteristically responding only by localized contraction of the spot stimulated, they exhibit generalized contractions only occasionally. If small enough, they progress by means of cilia; larger pieces do not progress at all. Sand is ingested and egested but burrowing is not effected. Longevity, in my specimens, was not as great as that of proboscis fragments, although Davydov (1908) and others have kept such fragments long enough for complete regeneration.

Thus, all regions possess the mechanism for nervous reflexes, and moreover, respond very largely by muscular contraction of very small areas immediately about the point of stimulation. It is not surprising, therefore, though crucially significant as an indication of the nervous pattern, to find that smaller and smaller fragments of the body wall are quite able to react nervously, the smallest piece that I could neatly cut and observe—having approximately 1 sq. mm. of surface—displaying nervous irritability and true reflexes. The evidences

against regarding this as direct muscular stimulation are: (1) the strength of adequate stimuli, which need be no greater than those required for indubitable, long distance reflexes in the intact animal whereas direct stimulation of muscles (thus in narcotized specimens) requires very strong stimuli, and (2) the reversible disappearance of the reflexes in light narcosis. Finally, it should be stated that fragments from any part of the surface of any region (except areas where the body wall is devoid of musculature) may thus react autonomously.

If small pieces of an animal, from any region, are able to execute normal reflexes, it is evident that the impulse does not need to travel to a distant central organ and then back to an effector, but that the entire path of the reflex is located within that small piece. Autonomy of this type is highly characteristic of nerve net-equipped organisms and its presence in balanoglossids is convincing evidence that the general superficial plexus of these animals is essentially a nerve net.

Polarization.—One of the earliest modifications of the primitive nerve net was in the direction of acquiring polarity. This means that impulses will travel more easily in one direction than in others, and will tend to spread differentially along an axis instead of uniformly in an expanding circle. It is of interest to look for evidence of polarity in the diffuse plexus of enteropneusts.

A proboscis cut into two pieces, transversely, exhibits no difference in the behavior of the two cut faces. The pieces themselves show no evidence of polarity aside from the direction of progression which is a result of the polarity of the ciliated field. It has already been shown that impulses spread in all directions from the point of stimulation in the proboscis.

The trunk exhibits an obvious polarity traceable to the nerve cords, but in the absence of these structures the lateral plexus shows little evidence thereof. Evidence has already been cited, however, which indicates a differential orientation of pathways such that impulses pass easily in the dorso-ventral direction, but only with difficulty longitudinally. No experiments have been devised to show that these two axes are heteropolar, although it is to be expected from other, already cited facts. The poles of the dorso-ventral axis would be the dorsal and ventral cords. These differ in several ways, not only histologically, but functionally, thus in respect to passage of impulses across the midline and in relative importance for the normal responses of the intact animal. Along the anteroposterior axis the usual direction of propagation of waves of contraction is from anterior to posterior. How much this normally depends on the cords cannot yet be

said, but it is possible that a degree of anteroposterior polarity exists in the plexus.

Action of Drugs

Unfortunately, almost no opportunity has been found to experiment with drugs whose effect on the nervous systems of higher animals is known. But two observations may be mentioned as of some potential interest.

The anaesthetic action of $MgSO_4$, chloretone, and cocaine has been frequently employed in other connections. The sequence of events in this action is suggestive of a distinction between two categories of nervous mechanisms. Whole specimens or fragments were exposed to these agents and tested from time to time for their responses to mechanical stimulation. It was found that local responses of the area immediately about the spot stimulated persisted long after the cessation of more general ones, involving major conduction paths.

The only other drug whose action on the nervous system has been tested is strychnine. Parker (1919), discussing some experiments of Moore (1917), suggests that this drug might afford a physiological test for the presence of synapses. Moore had found that strychnine exerted no effect on certain coelenterates, a slight effect on some echinoderms, and a considerable one on certain crustacea and cephalopods, a series which leads nicely up to the well-known effect on vertebrates generally regarded as a heightening of irritability by decrease of synaptic resistance. Whole specimens of *Saccoglossus pusillus* and pieces of *Balanoglossus occidentalis*, immersed for periods up to several hours in concentrations of from 1 : 1000 to 1 : 100,000 of strychnine sulphate in sea water, showed no effect whatsoever. The responses appeared entirely normal in character, strength, and threshold of stimulation.

This experiment points to a condition similar to that of coelenterates and different from that of higher organisms. But, since the existence of synapses in coelenterates, long denied, has recently been claimed (Bozler, 1927; Hanström, 1928; Woollard and Harpman, 1939), this physiological difference cannot be safely interpreted as proving anatomical continuity. Nor can I follow Parker (1919) and Ariëns Kappers (1929) in assuming that the existence of a physiological nerve net, evidenced by the properties of diffuse transmission, autonomy, etc., necessarily means protoplasmic continuity of neurons. At present it cannot be said whether the physiological nerve net of balanoglossids is synaptic or asynaptic.

Summary

1. A picture of the functional plan of organization of the nervous system of balanoglossids, based on experiments with *Saccoglossus pusillus* and *Balanoglossus occidentalis*, is offered. The state of the receptor and effector mechanisms is also reviewed and correlated with the habit of life.

2. These animals are shown to be highly dependent on a general superficial ciliature whose complex behavior suggests the possibility of nervous control.

3. A great abundance of several types of integumentary glands capable of voluminous secretion, represents a second important effector system. Lacking any physiological evidence of the nature of the products and the variations in activity of the glands, nothing can yet be said regarding the relation of this apparatus to the nervous system.

4. Identification of the organs of light production has not been made. Crozier's argument for their independence from the nervous system is controverted but no positive statement of their nature and control can yet be made.

5. The muscular system is characterized by sluggish action and produces movements of a simple generalized character, lacking differentiation into a variety of reflexes. The paucity of clearly defined and diversified responses greatly limits the possibilities and conditions the nature of experiments on the nervous system.

6. The receptors, likewise, are at a low level of development, lower than that of many coelenterates, being represented by generalized, scattered sense cells. The mechanism of the photic response is discussed in the light of new experiments.

7. The nervous system is shown experimentally to be diffuse and superficial.

8. Properties characteristic of nerve nets—diffuse transmission, decremental conduction, and neuromuscular autonomy of every small fragment of body wall—are demonstrated to exist in balanoglossids. But the presumption of anatomical continuity of neurons from these physiological properties is not assumed, though the facts point to relations resembling those of coelenterates and differing from those of higher animals.

9. The physiological nerve net has been modified correlative with the elongated, bilaterally symmetrical body and the development of great conduction paths in the mid-dorsal and mid-ventral lines. Conduction through the general plexus is more easily induced in the transverse plane than longitudinally.

10. Specialized conduction tracts are demonstrated which correspond to the known histologic concentrations of punctate tissue.

11. Of these, the ventral tract in the trunk is less dispensable than the dorsal, but the latter may be slightly more specialized.

12. Almost no suggestions have been found that these cords, including the internal nerve cord of the collar, function in a ganglionic or central nervous capacity. They are all primarily conduction paths.

13. These facts, together with the general behavior of the animals and the high degree of autonomy of parts are considered to render inappropriate the designation "central nervous system" for the collar nerve cord or any or all of the concentrations of nerve tissue in balanoglossids.

14. The picture of the nervous mechanism of the Enteropneusta is that of an exceedingly primitively organized system. This must be emphasized, but is not considered to affect, one way or the other, the morphologic arguments concerning the relations of these animals and the chordates.

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THE EFFECTS OF VANADIUM, COPPER, MANGANESE AND IRON ON THE SYNTHESIS OF PROTOPLASM BY CHILOMONAS PARAMECIUM [1]

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About forty years ago it was believed that only ten elements are necessary for the synthesis of living matter—carbon, oxygen, hydrogen, nitrogen, phosphorus, sulfur, calcium, magnesium, potassium and iron. It was known before then that many heavy metals besides iron are found in living matter but it has been only during recent years that their indispensability in some organisms and beneficial action in others have been demonstrated. Vanadium, copper, and manganese are among these; however, practically nothing has been done concerning the effect of these elements on the protozoa.

An excellent opportunity to make such a study is offered by the colorless biflagellate protozoan, *Chilomonas paramecium*, which can synthesize starch, fat, proteins and protoplasm from relatively simple compounds under environmental conditions which can be accurately controlled. The results presented in the following pages were obtained in a study of the effects of vanadium, copper, manganese and iron on the rate of reproduction of this organism.

Material and Methods

The original specimens of *Chilomonas paramecium* used in these experiments were obtained from a strain which has been maintained in the Zoölogical Laboratory of The Johns Hopkins University for eight years, at first, in sterile acetate-ammonium solution (Mast and Pace, 1937) and lately in a sterile modified acetate-ammonium solution (Table I). The modified acetate-ammonium solution will be referred to as the acetate-ammonium solution.

Kahlbaum chemicals "highest purity" were used exclusively. All water used in the preparation of solutions was redistilled in a tandem Pyrex glass still (Mast, 1928). Chemically clean Pyrex glass double

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depression slides, Erlenmeyer flasks, and pipettes were used throughout in the experimental work.

Tests were made to ascertain whether the Kahlbaum salts used to prepare the acetate-ammonium solution contained manganese and ferric iron. The test used to detect manganese depends upon the formation of $KMnO_4$ from KIO_4 and salts of manganese when together in acid solution. By this method concentrations of manganese as low as 10^{-5} M can be detected. By using concentrated solutions of salts in which manganese cannot be detected, diluted 1000 times in making the acetate-ammonium solution, it is certain that the concentration of manganese in the culture medium did not exceed 10^{-8} M.

Two tests were used for iron, one based on the formation of $Fe_4[Fe(CN)_6]_3$ (Prussian blue) by ferric iron in the presence of $K_4Fe(CN)_6$ and the other on the formation of $Fe(CNS)_6^{---}$(ferric thiocyanate ion) by ferric iron in the presence of KCNS (Feigl, 1937; pp. 93 and 95). By these methods a concentration of iron as low as 5×10^{-5} M can be detected. By using concentrated solutions of salts in which iron cannot be detected, diluted 1000 times in making the acetate-ammonium solution, it is certain that the concentration of iron in the culture medium did not exceed 5×10^{-8} M. Kahlbaum contends, however, that the $CaCl_2$ used contained 0.0001 per cent iron. If this is true the concentration of iron was at least 1.98×10^{-10} M.

TABLE I

Composition of the modified acetate-ammonium solution used to culture *Chilomonas paramecium*. Hydrogen ion concentration adjusted to pH 6.8 by means of HCl.

Chemicals	Mgm. per 100 cc. Water	Molar Concentrations
$NaC_2H_3O_2$	150.00	0.011
K_2HPO_4	20.00	0.0011
NH_4Cl	46.00	0.0086
$(NH_4)_2SO_4$	10.00	0.00076
$CaCl_2$	1.16	0.00010
$MgCl_2$	1.00	0.00010
HCl	Variable	Variable

The effects of the heavy metals on *Chilomonas* were ascertained by comparing the growth of isolated specimens in acetate-ammonium solution to which varying amounts of compounds of heavy metals were added with the growth of specimens in acetate-ammonium solution to which none were added.

The solutions containing various amounts of heavy metals were prepared as follows: To 49.5 cc. of acetate-ammonium solution in a chemically clean 125 cc. Pyrex glass Erlenmeyer flask enough of the compound of the heavy metal in 0.5 cc. of solution was added to yield

50 cc. of solution containing the highest concentration tested. Then by serial dilution, 50 cc. of each concentration of the metal being tested was prepared in acetate-ammonium solution in individual 125 cc. Erlenmeyer flasks.

The hydrogen ion concentration of these solutions, except in the higher concentrations of the compounds of the heavy metals, was pH 7.2. Since *Chilomonas* grows best at pH 6.8 to 6.6 (Mast and Pace, 1938), enough HCl was added to increase the hydrogen ion concentration to pH 6.8. The amount of HCl needed varied with the kind and concentration of the compound of the heavy metal in the solution. The highest concentration of $VOCl_2$ required none; the highest concentration of Na_3VO_4 required 0.1 cc. 0.4 M; and the other high concentrations and all low concentrations required amounts varying between these. The hydrogen ion concentration was measured daily and maintained at pH 6.8 to 6.6 throughout each experiment.

After the solutions were prepared, the flasks were stoppered with non-absorbent cotton, heated in an oven to 80° C. and kept at this temperature for about 20 minutes. This heating was repeated daily, resulting in solutions which were always entirely free of bacteria and molds.

The experiments were performed as follows: Two dry clean double depression slides were put into each of a number of Petri dishes, heated to 135° C. and left at least 30 minutes. After cooling, each Petri dish was labeled to indicate the kind of solution which was to be put into it, and then 0.1 cc. of that solution put into each of the four depressions. (A different pipette was used for transferring each solution.) One chilomonad was then transferred to each depression in all Petri dishes, directly from a sterile clone which had been growing in acetate-ammonium solution for two or three days. The Petri dishes were then put into an incubator in which the air was very humid and the temperature 24 $\pm$ 0.5° C. After 24 hours the number of divisions that had occurred in each depression was recorded and one individual from each depression was transferred to another depression containing fresh solution of the kind from which it had been taken. The specimens which were not transferred were either discarded or stained to ascertain the amount of starch and fat in them. This procedure was repeated daily as long as the experiment was continued.

Vanadium

Vanadium is usually thought of as a rare metal. This, the evidence shows, is hardly true. It occurs in the crust of the earth to the

extent of 0.017 per cent, whereas copper, zinc, and lead occur respectively to the extent of only 0.0104, 0.0039 and 0.0020 per cent (Marks, 1929).

The occurrence of vanadium in living systems is rare, but it has been known for a long time to occur in plants. According to Suzuki (1903), Lippmann found it in 1888 in the ash of sugar beets, and Demarcay (1900) found it in the wood of oak, elm, poplar and pine trees, and the grape vine.

Henze (1932) found vanadium in 1911 in the blood corpuscles of the ascidian, *Phallusia mammillata*. Vinogradov (1930) found it in *Phallusia obliqua* and remarked that this species is so numerous along the shores of the Russian Arctic Sea that a great accumulation of vanadium now exists there. Vinogradov postulated that the metal functionally takes the place of iron and copper in the blood of the ascidians, implying that it functions in the carrying of oxygen. Henze presented evidence against this view and suggests that the divalent vanadium of the ascidian blood in some way serves as a reducing agent in the formation of the cellulose in the tunic.

Experimental Procedure

The effects of partially reduced vanadium (tetravalent), in $VOCl_2$, and of oxidized vanadium (pentavalent), in Na_3VO_4, were ascertained as follows: Varying concentrations of each were prepared in acetate-ammonium solution, the hydrogen ion concentration adjusted, the solution kept free of bacteria and molds and the experiments made as described above.

Seven experiments were made to ascertain the effect of $VOCl_2$. Of these experiments one was continued 26 days, one 40 days, two 12 days, two 14 days and one 15 days. The chilomonads used in all except one of the experiments were selected from ordinary vigorous cultures. Those used in this experiment were obtained from a culture produced by continuously selecting slow growing specimens for many generations. The former divided considerably more frequently than the latter under identical conditions.

In the former experiments the four original lines in each concentration were continued for the duration of the experiment, but in the latter new lines were started every eight days from a line that had divided regularly throughout the preceding eight days, twice in acetate-ammonium solution containing no vanadium and twice in acetate-ammonium solution containing an optimum concentration of $VOCl_2$. The latter were washed free of vanadium before transferring.

Three experiments were made to ascertain the effect of Na_3VO_4.

Two were continued 15 days and one 12 days. In each the chilomonads used were selected from ordinary vigorous cultures.

The results obtained are presented in Tables II and III and in the following paragraphs.

Results

Table II shows that in experiment I as the concentration of $VOCl_2$

TABLE II

Effect of partially oxidized vanadium (tetravalent), in $VOCl_2$, in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature, 24 ± 0.5° C.

Molar Concentrations of $VOCl_2$	Average Number Divisions per Day of Four Lines	
	Experiment I (26 days)	Experiment II (40 days)
0	3.19±.040*	2.20±.033*
10^{-7}	3.33±.054	2.39±.027
10^{-6}	3.58±.051	2.43±.029
4×10^{-6}	3.58±.064	———
5×10^{-6}	———	2.43±.029
7×10^{-6}	3.38±.063	———
10^{-5}	3.65±.041	2.42±.029
4×10^{-5}	3.67±.039	———
5×10^{-5}	———	2.54±.029
7×10^{-5}	3.61±.054	———
10^{-4}	3.33±.048	2.33±.030
3×10^{-4}	2.97±.086	———

* Probable errors.

increased the rate of reproduction in *Chilomonas* increased from 3.19 divisions per day in acetate-ammonium solution with no vanadium to 3.67 at 4×10^{-5} M and then decreased to 2.97 at 3×10^{-4} M, and that in experiment II the rate of reproduction increased from 2.20 divisions per day in acetate-ammonium solution with no vanadium to 2.54 at 5×10^{-5} M and then decreased to 2.33 at 10^{-4} M. These results show then that partially reduced vanadium (tetravalent), in $VOCl_2$ at certain concentrations, is definitely beneficial for growth in *Chilomonas* and that the optimum concentration is between 10^{-5} M and 7×10^{-5} M.

In some of the experiments which were continued for shorter periods the concentration of $VOCl_2$ was lower than 10^{-7} M and in others it ranged from 10^{-7} M to 10^{-4} M. No verifiable acceleration of growth was obtained in the experiments with concentrations lower than 10^{-7} M, but definite acceleration was obtained at approximately 10^{-5} M in the other experiments.

TABLE III

Effect of oxidized vanadium (pentavalent), in Na_3VO_4, in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature, 24 ± 0.5° C.

Molar Concentrations of Na_3VO_4	Average Number Divisions per Day of Four Lines (15 days)
0	3.76±.063*
10^{-12}	3.74±.057
10^{-10}	3.68±.054
10^{-8}	3.59±.049
10^{-7}	3.73±.045
10^{-6}	3.52±.047
10^{-5}	3.65±.051
10^{-4}	3.63±.052
10^{-3}	3.50±.044

* Probable errors.

Table III shows that there was no increase in rate of reproduction of *Chilomonas* in acetate-ammonium solution containing oxidized vanadium (pentavalent), in Na_3VO_4, but that in the highest concentration tested the rate of reproduction was considerably lower than in the

TABLE IV

Effect of Na_3VO_4 and NaCl in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature 24 ± 0.5° C.

Molar Concentrations of Sodium	Average Number Divisions per Day of Four Lines (7 days)	
	Na_3VO_4	NaCl
0	3.34±.067*	3.34±.067*
3×10^{-3}	3.00±.093	3.28±.060
3×10^{-7}	3.35±.082	3.37±.071

* Probable errors.

acetate-ammonium solution. This experiment was repeated several times. Essentially the same results were obtained in all. Since a molecule of Na_3VO_4 contains three atoms of sodium and only one of vanadium, the question arises whether the inhibition in the rate of growth was due to the sodium or to the vanadium. To answer this question *Chilomonas* was grown in acetate-ammonium solution and in this solution which contained respectively Na_3VO_4 and NaCl in such quantities that the concentration of sodium was the same in both. The results obtained are given in Table IV.

Table IV shows that during seven successive days the average

number of divisions per day in acetate-ammonium solution containing 10^{-3} M Na_3VO_4 was 0.34 of a division less than in the acetate-ammonium solution with no Na_3VO_4 or NaCl, and that in the solution containing 3×10^{-3} M NaCl the average was only 0.06 of a division less. It also shows that the frequency of division in the solution containing 10^{-7} M Na_3VO_4 and in that containing 3×10^{-7} M NaCl was essentially the same as in the acetate-ammonium solution. These results demonstrate therefore that the decrease in the rate of growth in 10^{-3} M Na_3VO_4 was due to the vanadium and not to the sodium in it. This conclusion is supported by the rate of reproduction in 3×10^{-4} M $VOCl_2$ (Table II).

Discussion

The fact that *Chilomonas* can tolerate Na_3VO_4 in concentrations as high as 10^{-3} M indicates that vanadium in this form is not toxic for protozoa. This conclusion is supported by the results obtained by Proescher and Seil (1917). They found that *Opalina ranarum* can tolerate concentrations of $Na_2V_4O_{11}$ as high as 0.284 M.

In other organisms vanadium has been found to be beneficial or detrimental depending on the concentrations. Suzuki (1903) showed that low concentrations of VSO_4 cause increase in the rate of growth of the roots of barley plants but not the total growth and that high concentrations cause cessation of growth. Scharrer and Schropp (Willis, 1936) found that in high concentrations of $NaVO_3 \cdot H_2O$ plant growth is retarded, if not completely stopped, but that in low concentrations there are indications of acceleration, and Shibuya and Saeki (Willis, 1936) report acceleration of plant metabolism by vanadates. Proescher and Seil (1917) report only injurious effects of vanadium on wheat seedlings.

Konishi and Tsuge (1933) studied the elements in soils in which the nitrogen-fixing bacterium *Azotobacter chroococcum* grows. They found by spectroanalysis that vanadium is always present in soil in which *Azotobacter* makes good growth, and that if either VCl_2 or $NaVO_3$ is added in concentrations of either 10^{-4} or 10^{-5} M to an artificial culture medium the amount of nitrogen fixed is five to ten-fold greater than that fixed in culture medium containing no vanadium.

Copper

According to Elvehjem (1935) Meissner first demonstrated, in 1817, that copper occurs in plants. It was not until about a century later that its general occurrence in plants was established by Guerithault (1920), Maquenne and Demoussy (1920) and others. These workers found considerable amounts in fresh plant tissues and seeds. Ma-

quenne and Demoussy found it in so many plants that they suggested that it must be essential in plant growth. Since then numerous investigators (Felix, 1927; Allison, Bryan and Hunter, 1927; Orth, Wickwire and Burge, 1934) have demonstrated that many soils are improved for plant growth by the addition of $CuSO_4$, and Sommer (1931) demonstrated that the element is necessary for plant growth. More recently, Kubowitz (1937) has shown that the polyphenol oxidase of the potato is a copper-containing protein. This is a possible explanation of the necessity of copper for plant growth.

Raulin (1869) believed that probably other metals besides iron and zinc are necessary for the growth of *Aspergillus niger*. Ono (1902) confirmed this belief when he obtained greater dry weights of *Aspergillus niger* by adding small amounts of copper to the culture solution. Lepierre (1913) maintains that zinc in Raulin's solution can be replaced, at least partially, by copper.

Up until recent years only two natural occurrences of copper in animals were known—in hemocyanin and in turacin, the red pigment of the feathers of the Turaco birds of South Africa. Thudichum (1901) claimed that he found it in the human brain, but his claim was not accepted until Bodansky (1921) found considerable amounts in the brains of four people. Now, because Hart, Steenbock, Waddell and Elvehjem (1928) demonstrated that highly purified iron salts are effective in correcting a deficiency of hemoglobin only when small amounts of copper are present, it is recognized that it plays an important rôle in the life of mammals.

Experimental Procedure

The effect of copper in $CuCl_2$ on the growth of *Chilomonas* was tested. Acetate-ammonium solution containing $CuCl_2$ in various concentrations was prepared, the hydrogen ion concentration adjusted and maintained, the solutions sterilized and the experiments made as described above. The results obtained are given in Table V.

Results

By referring to Table V it will be seen that in acetate-ammonium solution containing concentrations of $CuCl_2$ of 10^{-12} M and less, the frequency of division was essentially the same as in acetate-ammonium solution with no copper (3.76 divisions per day) and that in concentrations higher than 10^{-12} M the frequency of division decreased as the concentration of $CuCl_2$ increased, division ceasing entirely at 3×10^{-7} M. In 3×10^{-7} M the chilomonads divided and lived five or six days. As the concentration increased from this the length of life decreased until at 10^{-6} M they lived only a few minutes.

Discussion

The results concerning the toxicity of copper are in accord with those obtained by others. Nägeli (1893) found that copper kills algae in extremely low concentrations. Seybold (1927) found that *Euglena* lives in 10^{-8} M $CuSO_4$ as long as food lasts, but that in 10^{-7} M it dies in less than half an hour.

Chalkley and Voegtlin (1932) found that $CuCl_2$ in a concentration as low as 2×10^{-8} M kills *Amoeba proteus* and that 2×10^{-9} M depresses growth, increases the rate of mortality, and probably decreases the rate at which food in the food vacuoles is digested and assimilated.

TABLE V

Effect of copper in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction in *Chilomonas paramecium*. Temperature $24 \pm 0.5°$ C.

Molar Concentrations $CuCl_2$	Average Number Divisions per Day of Four Lines (15 days)
0	3.76±.046*
10^{-20}	3.70±.056
10^{-18}	3.74±.062
10^{-16}	3.76±.051
10^{-14}	3.76±.051
10^{-12}	3.79±.045
10^{-10}	3.60±.056
10^{-8}	3.42±.072
10^{-7}	3.24±.088
3×10^{-7}	died
5×10^{-7}	"
8×10^{-7}	"
10^{-6}	"

* Probable errors.

Sommer (1931) found that flax, sunflower and tomato plants mature normally when grown in artificial nutrient medium containing copper but do not if it does not contain copper, and Ono (1902), Bortels (1927) and Roberg (1928) showed that small amounts of copper cause an increase in the total growth of *Aspergillus niger*. This indicates that copper is beneficial for growth in many different organisms.

The fact that the rate of reproduction in *Chilomonas* was no higher in any of the concentrations of copper tested than in acetate-ammonium solution without copper indicates that copper is not beneficial for reproduction in this form. The facts that it has been grown for eight years in this laboratory in solutions composed of chemicals of the highest purity with no copper added and that the water in these

solutions was triply distilled in a tandem Pyrex glass still and was kept in Pyrex glass containers, i.e. in glass which according to the makers contains no copper (communication from the Corning Glass works) strongly indicate that copper is not needed for growth in *Chilomonas*.

Manganese and Iron

McHargue (1926) says that in 1788 Scheele showed that pyrolusite (MnO_2) is the oxide of manganese which was then a new metal and that manganese is assimilated by plants. Manganese has since been found in so many plants by Maumené (1884), Headden (1915) and Bertrand and Rosenblatt (1921) that it is now believed to be in all (Miller, 1931).

The addition of manganese to soil was early found to be beneficial to the growth of plants (Nagaoka, 1903; Bertrand, 1905; Brenchley, 1910; McHargue, 1926; and Bryan, 1929). Bertrand (1905) obtained such poor growth in plants with deficiencies of it that he suspected it to be indispensable for growth. This was proved to be true by McHargue (1926), Gilbert and Pember (1931), and McHargue and Calfee (1932).

According to Oettingen (1935) manganese has been found in the blood, the liver and the kidneys in relatively large quantities and in lesser quantities in all other tissues in animals. Its functional rôle in animals is, however, not definitely known. Some investigators (Titus, Cave and Hughes, 1928; Myers and Beard, 1931) found it beneficial in blood regeneration and others (Waddell, Steenboch and Hart, 1929; Orent and McCollum, 1931; and Mitchell and Miller, 1931) found it either non-beneficial or only negligibly beneficial. Orent and McCollum found that rats fed on a diet free of manganese grow to maturity in an apparently normal manner, but that their offspring are undersized and inferior in appearance and that they are not suckled by their mothers. They also found that male rats raised on such a diet develop normally but have degenerated testes.

Gris discovered in 1845 that iron is a constituent of chlorophyll-bearing plants (Bortels, 1927). Since then it has been established that green plants which are deprived of it become chlorotic due to the absence of chlorophyll (Miller, 1931). Raulin (1869) found that if iron is added to culture media in which *Aspergillus niger* is growing the total weight produced is greatly increased.

Iron is found in nearly all if not all animal tissues. It is in all red blood corpuscles and Jones (1920) found it in the nuclei of cells of the liver and kidney and in the cytoplasm and the nuclei of cells of the spleen in adult and foetal guinea pigs. He found it in various tissues of the sparrow, frog, fish, crayfish, oyster, earthworm and

hydra, and says that staining reactions for it are stronger in lower animals than in higher and in foetal mammalian tissues than in adult.

It is well known that iron is closely associated with the transfer of oxygen by blood. In plants it is thought to act catalytically in the formation of chlorophyll (Wolff, 1913). Warburg (1925) contends that in all cells it functions mainly in respiration and that this is due to the catalytic action of a hematin derivative.

Experimental Procedure

The effect of manganese in $MnCl_2$ and iron in $FeCl_3$ on the growth of *Chilomonas* was tested in accord with the preceding experiments on vanadium and copper. The results obtained are presented in Table VI.

TABLE VI

Effect of manganese and iron in different concentrations in acetate-ammonium solution (Table I) on the rate of reproduction of *Chilomonas paramecium*. Temperature 24 ± 0.5° C.

$MnCl_2$ and $FeCl_3$ Added; Molar Concentrations	Average Number Divisions per Day of Four Lines	
	$MnCl_2$ (19 days)	$FeCl_3$ (17 days)
0	3.28±.055*	3.30±.045*
10^{-9}	3.25±.045	3.38±.046
5×10^{-9}	3.17±.063	3.37±.045
10^{-8}	3.31±.058	3.37±.041
5×10^{-8}	3.39±.049	3.40±.045
10^{-7}	3.33±.048	3.33±.049
5×10^{-7}	3.40±.046	3.39±.042
10^{-6}	3.25±.041	3.33±.057
5×10^{-6}	3.28±.051	3.47±.045
10^{-5}	3.38±.042	not tested
5×10^{-5}	3.28±.049	" "
10^{-4}	3.39±.041	" "

* Probable errors.

Results

Table VI shows that in the solutions to which $MnCl_2$ was added the frequency of division varied from 3.17 to 3.40 per day and that in those to which $FeCl_3$ was added it varied from 3.33 to 3.47 per day, but that there is no indication of any correlation between the concentration of either manganese or iron and the frequency of division and no statistically significant difference between the rate of reproduction in the solutions to which iron and manganese were added and the acetate-ammonium solution. These tests were repeated and essentially the same results were obtained. They therefore show that iron and manganese neither augment nor retard growth in *Chilomonas*.

Cultures of *Chilomonas* have been maintained in this laboratory in acetate-ammonium solution for nearly eight years without any indication of loss of vigor. Neither iron nor manganese was added to the solution. It consequently appears that neither is necessary for growth in *Chilomonas*. Kahlbaum maintains, however, that the $CaCl_2$ used in the solution contains 0.0001 per cent iron and some of the salts used may have contained a trace of manganese. The fact that *Chilomonas* grows in acetate-ammonium solution does not therefore prove that iron and manganese are unnecessary, but the fact that neither was detected in the tests described above which are sensitive to 5×10^{-8} M for iron and 10^{-8} M for manganese demonstrates conclusively that if these metals are necessary extremely minute quantities suffice.

Discussion

Molisch (1894) grew *Aspergillus niger* and *Penicillium* sp. in a culture medium to which no iron was added. However, analysis showed that they contained iron, and when iron was added to the culture medium the amount of growth was much greater. Molisch therefore concluded that these two molds need iron. The conclusion that *Aspergillus* needs iron has been amply verified by Sauton (1911), Steinberg (1935), Bortels (1927) and Roberg (1928). Sauton also found that *Penicillium* needs iron, but that the molds *Mucor mucedo*, *Rhizopus nigricans* and *Racoelium coellare* do not need it.

Bertrand and Javillier (1911) found that the dry weight of *Aspergillus niger* increased as the manganese content increased to 1000 mg. per 100 cc. and then decreased. Later Bertrand (1912) obtained augmentation of a crop of mold by the addition of an amount of manganese equivalent to one mg. per 10,000 liters of nutrient medium and because of this evidence suggested a catalytic rôle of the metal.

Hotchkiss (1923) found that iron does not accelerate the growth of *Bacterium coli*, although it has been found to accelerate the growth of many bacteria. Koser, Finkle, Dorfman and Saunders (1938) tested the possibility that inorganic salts present as impurities in preparations of spleen, liver, and yeast cause the growth-promoting activity of these preparations. They found that salts of neither iron, manganese nor copper were responsible for the growth in five bacteria and a yeast caused by additions of the preparations; however, they point out that their results do not unequivocally invalidate the evidence of the numerous investigators who have found that small amounts of the heavy metals do accelerate cell reproduction.

Hall (1937) maintains that manganese causes acceleration in growth in *Euglena anabaena*, but not in *Colpidium campylum* and *Astasia*.

These studies show that, although the heavy metals cause acceleration of growth in many organisms, they do not do so in all, and they seem to be indispensable to some forms and not to others. As already stated, the results of Tables V and VI show that the growth of *Chilomonas* is not accelerated by additions of copper, manganese and iron, and they indicate that these metals are not indispensable to *Chilomonas*. More evidence, however, is highly desirable.

Some evidence concerning the necessity of iron is found in the results obtained in the study of respiration in *Chilomonas*. If *Chilomonas* respires by means of an enzymatic iron-porphyrin system, the most common of which is that involving cytochrome and indophenol oxidase, iron is obviously necessary. Cytochrome, a hemochromogen, and indophenol oxidase are found in most aerobic organisms and tissues, including mammalian tissues, plants, insects, yeasts and aerobic bacteria (Meldrum, 1934). However, tests of a heavy suspension of chilomonads by means of a hand spectroscope and a microspectro-

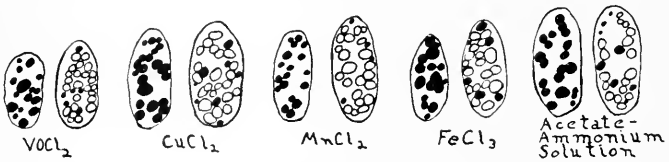

FIG. 1. Camera outlines showing the variation in starch and fat content in specimens of *Chilomonas paramecium* in acetate-ammonium solution (Table I) and in this solution with vanadium (5×10^{-4}M), copper (10^{-10}M), manganese (5×10^{-5}M) and iron (10^{-5}M) added respectively. ○, starch; ●, fat. Note that the starch and fat content varied from no starch and much fat to much starch and little fat and that this occurred in specimens grown in acetate-ammonium solution as well as in this solution with metals added.

scope revealed no absorption bands characteristic of cytochrome, and Hutchens (1939), working in this laboratory, reports that the respiratory system of *Chilomonas* is not sensitive to cyanide, which indicates the absence of indophenol oxidase and cytochrome.[2]

Relation between Vanadium, Copper, Manganese and Iron and the Amount of Starch and Fat in Chilomonas

From time to time chilomonads grown in acetate-ammonium solution and in this solution containing the various metals under consideration were studied under the microscope to ascertain the effect of the metals on the starch and fat content. This was done as follows:

[2] Since this paper went to press, Hutchens, by personal communication, has reported the presence of cytochrome and a sensitivity of respiration to cyanide in *Chilomonas* when grown in a more complex medium in which greater numbers of organisms are obtained.

Equal numbers of specimens were selected at random from each of the four lines in a given solution and put into a vaseline ring on a glass slide with as little water as possible; then lugol solution was added and a few minutes later sudan III. This fixed the chilomonads and stained the starch and fat in them. Specimens representing the extent of variation in fat and starch content were then selected and camera outlines made. One of the specimens in each solution which contained the greatest amount of starch and one which contained the greatest amount of fat are presented in Fig. 1.

This figure shows that the relative content of starch and fat varied enormously in different specimens, but that it varied just as much in those grown in acetate-ammonium solution as in those grown in this solution containing any one of the metals used and that there is no correlation between this variation and the kind or the concentration of these metals.

Observations on the relation between the rate of reproduction of the chilomonads in these solutions and the starch and fat content show that the fat content varied inversely and the starch content directly with the rate of reproduction in all of the solutions, the acetate-ammonium solution as well as this solution plus the metals. The relative amount of starch and fat in *Chilomonas* is therefore correlated with the rate of reproduction.

Summary

1. Experiments were performed to ascertain the effect of vanadium ($VOCl_2$ and Na_3VO_4), copper ($CuCl_2$), manganese ($MnCl_2$) and iron ($FeCl_3$) in acetate-ammonium solution on the rate of reproduction and the synthesis of starch and fat in *Chilomonas paramecium*.

2. Tetravalent vanadium in $VOCl_2$ causes marked increases in the frequency of division in *Chilomonas* which vary with the concentration. As the concentration of $VOCl_2$ added to the acetate-ammonium solution was increased, the frequency of division increased to a maximum at approximately 10^{-5} M and then decreased. Pentavalent vanadium in Na_3VO_4 causes no increase in frequency of division. Tetravalent vanadium at certain concentrations is therefore beneficial for growth of *Chilomonas* and pentavalent vanadium is not.

3. Neither copper, manganese nor iron in acetate-ammonium solution causes any statistically significant increase in the frequency of division of *Chilomonas*, and neither manganese nor iron causes a significant decrease in the frequency of division, but as the concentration of copper increases from 10^{-12} M the frequency of division decreases, ceasing entirely at 3×10^{-7} M.

4. *Chilomonas* has been grown continuously for eight years in acetate-ammonium solution with no additions of copper, manganese or iron. This shows conclusively that if the metals are needed extremely minute quantities suffice, but it does not prove that they are unnecessary for there are doubtlessly traces of them in the solution.

5. The starch and fat content of *Chilomonas* varied from no starch and much fat to much starch and little fat in all the solutions used; therefore, this variation was not caused by the addition of either vanadium, copper, manganese or iron. It was found in all solutions to be correlated with the rate of reproduction.

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STUDIES ON THE TREMATODES OF WOODS HOLE. III. THE LIFE CYCLE OF MONORCHEIDES CUMINGIAE (MARTIN) WITH SPECIAL REFERENCE TO ITS EFFECT ON THE INVERTEBRATE HOST

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This work was done during the several past summers spent at the Marine Biological Laboratory. A synopsis of this research was given before the 1939 Columbus, Ohio meeting of the American Society of Parasitologists. *Monorcheides cumingiae* parasitizes the intestines of flounders and eels, two important food fishes, and so may be of some economic importance. Very little work, particularly of an experimental nature, has been done on the life cycles of the members of the family *Monorchidae* to which this species belongs.

The family name *Monorchidae* was erected by Odhner (1911) with *Monorchis monorchis* (Stossich) as the type genus and species. As the family name indicates, one testis is characteristic of the group, but the genus *Monorcheides* is an exception in that two testes are present. The genus *Monorcheides* was created by Odhner (1905) for some small worms found in the intestine of the marine fish, *Lumprenus medius*, collected along the west coast of Spitzbergen.

Although the members of this family are rather widely distributed, very little work has been done to trace the biological relationships of the stages in the life cycles. According to Yamaguti (1938), Nagano (1930) found that a cercariaeum that developed in rediae and encysted in *Bulimus striatus japonicus* (Pilsbury) became the adult of *Asymphylodora tincae*. Yamaguti (1934) states that the species Nagano was working with was probably *A. macrostoma* Ozaki but in a later paper (1938) refers this form to *A. japonica* Yamaguti. Yamaguti (1934) reported finding what he believed to be the larval form of *A. macrostoma* Ozaki encysted in the peribuccal connective tissue of *Chaenogobius macrostomus*, although no feeding experiments were conducted. In 1938 this same author reports that on the basis of anatomical and ecological evidence a cercariaeum and its cyst found in *Bulimus striatus japonicus* (Pilsbury) probably represent stages in the life cycle of *Asymphylodora japonica* Yamaguti. In the same paper he

reported finding the larva of *Asymphylodora macrostoma* Ozaki encysted in the peribuccal connective tissue and gill arches of *Gnathopogon elongatus caerulescens* (Sauvage) from Lake Biwa and from *Cobitus biwae* Jordan and Snyder from the Katura River, Japan. During the past summer it was possible to demonstrate that *Cercaria cumingiae* Martin (1938), when fed under experimental conditions to flounders and eels, develops into an adult belonging to the genus *Monorcheides*.

In addition to the genus *Monorcheides*, the family *Monorchidae* includes species belonging to the following genera which have been collected from widely scattered regions: *Asymphylodora* Looss 1899 from Egyptian (Looss, 1899), Japanese (Yamaguti, 1938), and Indian (Srivastava, 1939) waters; *Bivesicula* Yamaguti 1934 from Japan; *Genolopa* Linton 1910 from Bermuda and Dry Tortugas (Linton, 1910), Beaufort, North Carolina (Manter, 1931), and North Queensland (Nicoll, 1915); *Hurleytrema* Srivastava 1939 from India; *Lasiotocus* Looss in manuscript and *Pristisomum* Looss in manuscript (Odhner, 1911) from Mediterranean and British (Nicoll, 1915) waters; *Monorchis* Looss 1902 from the Mediterranean Sea and British waters (Nicoll, 1915); *Paramonorcheides* Yamaguti 1938 from Japan and from the Galapagos Islands (Manter, 1940); *Paraproctotrema* Yamaguti 1934 from Japan; *Physochoerus* Poche 1925 from the Mediterranean Sea; *Proctotrema* Odhner 1911 from the Scandinavian arctic (Odhner, 1911), from Beaufort, North Carolina, Costa Rica, and the Galapagos Islands (Manter, 1931, 1940), from Japan (Yamaguti, 1934) and from the Karachi and Arabian Seas (Srivastava, 1939); *Proctotrematoides* Yamaguti 1938 from Japan; and *Telolecithus* Lloyd and Guberlet 1932 from Puget Sound and from Panama (Manter, 1940).

Materials and Methods

Living material, preserved whole mounts, and serial sections were used in this study. Living material is particularly useful in working out the finer details of the anatomy of the larval stages. Neutral red was used to stain the living material, while on preserved specimens Mayer's paracarmine was used on whole mounts and Delafield's hematoxylin and eosin were used on sectioned material. The mounting medium, "Clarite," was used and proved very satisfactory.

The mollusks, *Cumingia tellinoides*, were placed in finger bowls and daily observations were made to detect the presence of emerging cercariae. Infected clams were isolated individually and heavy infections of metacercariae were established in them. Heavy infections were produced also in clams that were not infected with sporocysts. In other words, some clams that had not served as first inter-

mediate hosts were used as second intermediate hosts. Infected clams were fed to flounders and eels that had been kept in aquaria for several weeks to allow the maturation of any worms that might have been present in the intestines of these fishes when they were brought into the laboratory.

Observations

The life cycle of *Monorcheides cumingiae* involves the development of sporocysts, cercariae, and metacercariae in the marine bivalve, *Cumingia tellinoides* (Conrad), and the development of the adult, under experimental conditions, in the intestines of eels and flounders.

The Life Cycle

The Sporocyst

The sporocysts are simple sac-shaped structures that develop in the visceral mass of the clam. Because of the large number of sporocysts within a single clam it seems probable that there is a daughter sporocyst generation although this has never been observed. The entire visceral region may be riddled with sporocysts, resulting in partial or complete castration and other disturbances. The intestine was usually surrounded by sporocysts and it is likely that this is the avenue of entrance of the miracidia. The fact that the eggs of this parasite failed to develop when kept for over three weeks in sea water suggests that they must be eaten by the clam in order for development to take place. The wall of the sporocyst is very thin and contains scattered nuclei. However, at the ends of the sporocysts thickened knobs of cells are present and they seem to take part in the formation of germ balls. The nuclei in these knobs enter protoplasmic extensions into the lumen of the sporocyst and, finally, actually leave the cytoplasm behind and take part in cercarial formation. The lumina of the sporocysts contain fragments, probably cellular, which stain with eosin.

The Cercaria

The cercaria bears two conspicuous eyespots in the anterior half of the body which still are evident in the recently encysted metacercaria (Fig. 5). The oral and ventral suckers are approximately equal in size. The excretory system is relatively simple and is represented by the formula $2[(2 + 2) + (2 + 2)]$. A mass of cells directly behind the ventral sucker represents the anlage of the reproductive system. The cercariae emerge from the clam by way of the excurrent siphon and swim about immediately. The light intensity is a controlling factor of the direction of swimming because the cercariae show a definite

negative response to light. Eventually the cercariae come in contact with the tissues of the clam, either by being forcibly taken in by the incurrent siphon or by coming in contact with the mantle or foot through their own swimming efforts. In both cases they soon shed their tails and work their way into the clam tissue where they encyst. Apparently, encystment does not take place prior to a period of swimming, i.e. the cercariae do not encyst in the time interval between emerging from the sporocysts and leaving the clam. The factor, or factors, controlling the time of encystment are unknown. The fact that a swimming period must occur before encystment takes place is of definite advantage to the parasite in increasing its range, since clams other than the infected one may become second intermediate hosts. In this connection it was found that another small clam, *Tellina tenera* Verrill, could serve as a second intermediate host. This same species may very rarely serve as the first intermediate host since one of several hundred examined was infected with sporocysts.

In the original description of *Cercaria cumingiae* Martin 1938, it was postulated that this species probably belonged to the family *Allocreadiidae* because of anatomical similarities to some of the cercariae of this family. However, the experimental results have disproved this postulation. Nevertheless, these similarities suggest a possible relationship of the family *Monorchidae* to the family *Allocreadiidae*.

EXPLANATION OF PLATES

Abbreviations used:

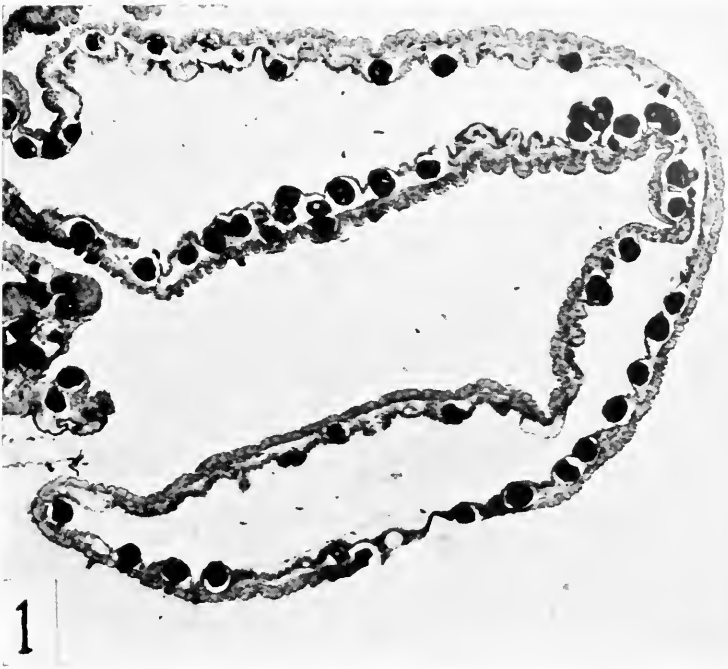
C.....cirrus	*OV*.....ovary
CS.....cirrus sac	*P*.....pharynx
E.....eyespot	*PR*.....prostate tissue
EB.....excretory bladder	*S*.....seminal vesicle
EG.....egg	*SP*.....spine
G.....genital pore	*T*.....testes
I.....intestine	*U*.....uterus
M.....metacercariae	*V*.....vagina
O.....oral sucker	*VI*.....vitellaria

Description of Plate I[1]

Fig. 1. Longitudinal section through the siphon of *Cumingia tellinoides* showing metacercariae just inside the muscular layer of the siphon.

Fig. 2. Section through the gill and part of the body of *Cumingia tellinoides* showing metacercariae in the gill and sporocysts (*a*) in the body.

[1] I wish to express my appreciation to Dr. E. J. Kohl of Purdue University for making the photomicrographs in Plates I and II.



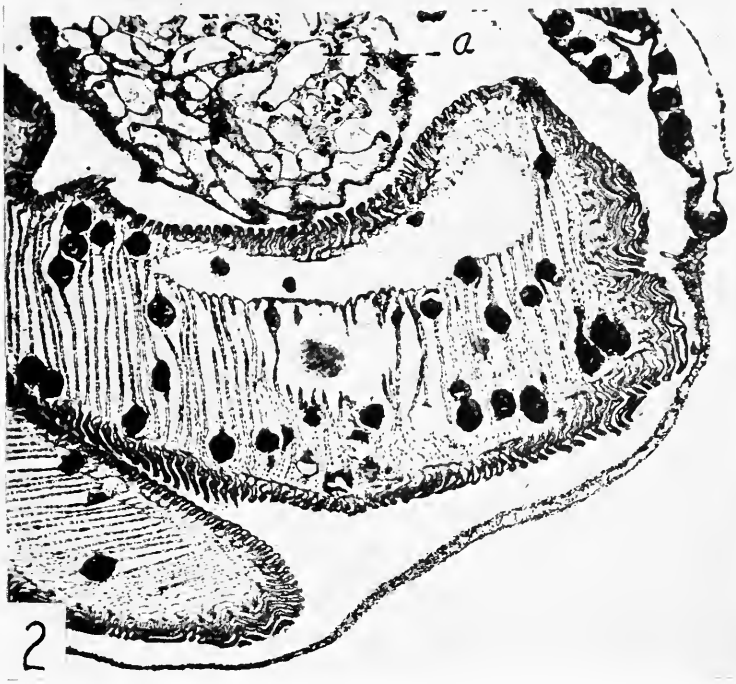


PLATE I

The Metacercaria

Upon shedding its tail and encysting the cercaria becomes a metacercaria. During the metacercarial stage certain important changes occur: the eyespots start to disintegrate, the digestive system becomes much better developed with the ceca reaching to near the posterior end of the body, there may be a slight increase in the number of cephalic glands, the excretory bladder loses its thick-walled appearance and becomes a thin sac due to the breaking down of the cells of the wall, there is an increase of two or three times in the size of the body, and the cuticula increases in thickness. A very interesting change occurs in the staining reaction of the nuclei with the aging of the metacercariae. The nuclei of recently encysted metacercariae can hardly be distinguished but as the metacercariae become older the nuclei stain heavily with Delafield's hematoxylin (Fig. 5). The significance of this change is unknown. During the metacercarial stage there is very little differentiation of the genital region.

The Adult (Figs. 6 and 7)

Under natural conditions, it seems probable that the definitive fish host obtains its infection by eating the siphons or the entire clam infected with metacercariae. *Cumingia tellinoides* and *Tellina tenera* extend their siphons a considerable distance beyond the edge of the shell and these structures could attract the attention of fishes. In the laboratory, small puffers, *Sphaeroides maculatus* (Schneider), were observed to bite off the siphons of *Cumingia* and other clams. In tracing this life cycle, entire infected *Cumingia* were removed from their shells and fed to three flounders and to three eels. Six of each species of fish were reserved as controls. With the exception of the infected *Cumingia*, both experimental and control fishes received the same type of food, namely clam meat, *Venus mercenaria*, *Mya arenaria*, *Modiolus modiolus*, and *Mytilus edulis*, which was examined carefully for any possible trematode infection. One of the eels, however, was

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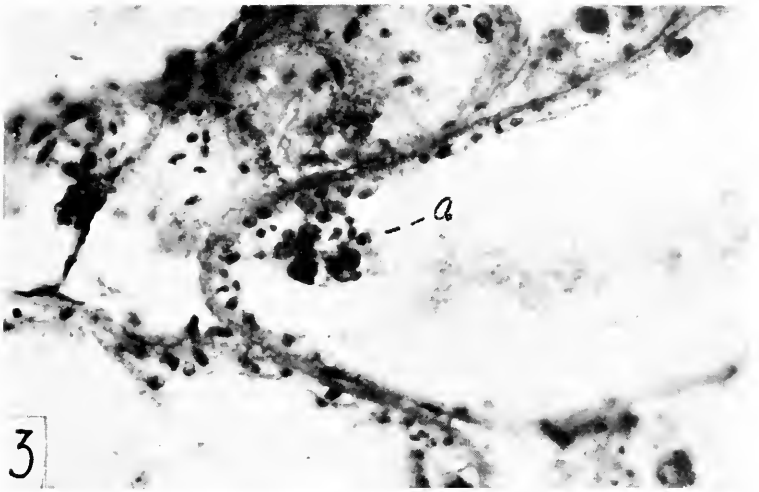
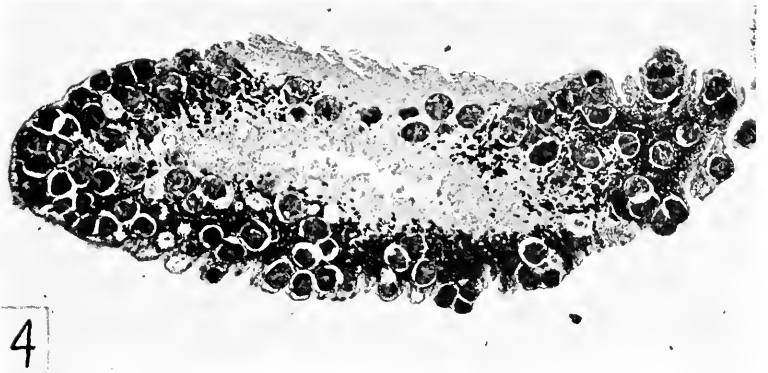
DESCRIPTION OF PLATE II[1]

FIG. 3. Section through a sporocyst showing the enlarged knob (*a*) of cells from which nuclei proliferate to form germ balls which become cercariae.

FIG. 4. Section through the foot of *Cumingia tellinoides* showing the metacercariae and the darkly staining material deposited around them by the clam.

FIG. 5. Section showing a recently encysted metacercaria (*a*) with conspicuous eyespots but showing no nuclei in contrast to older, adjacent metacercariae whose nuclei stain heavily.





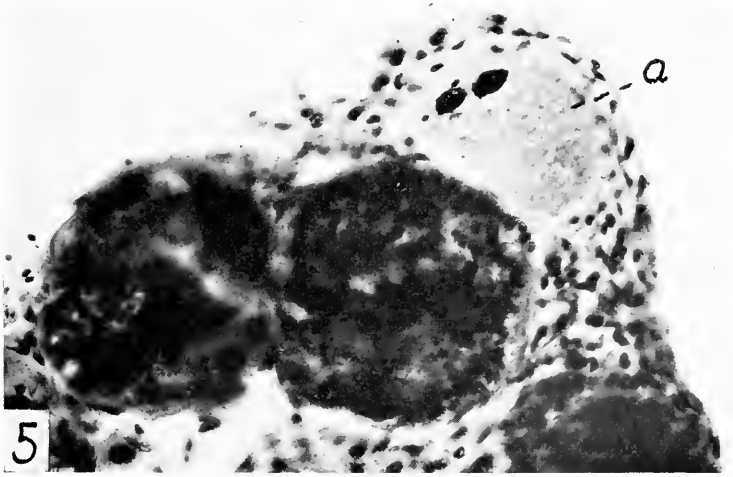
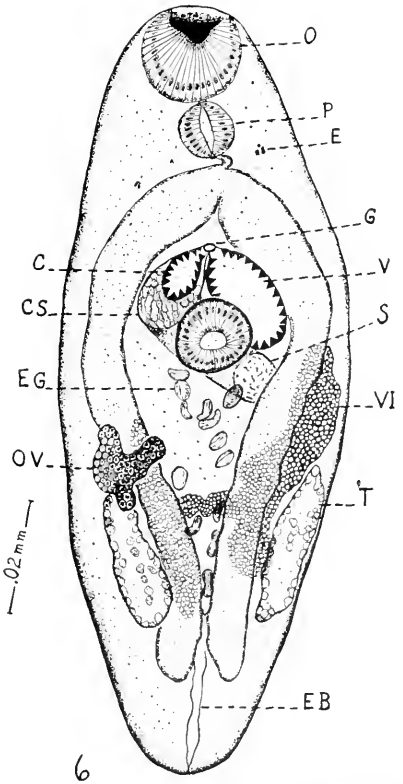
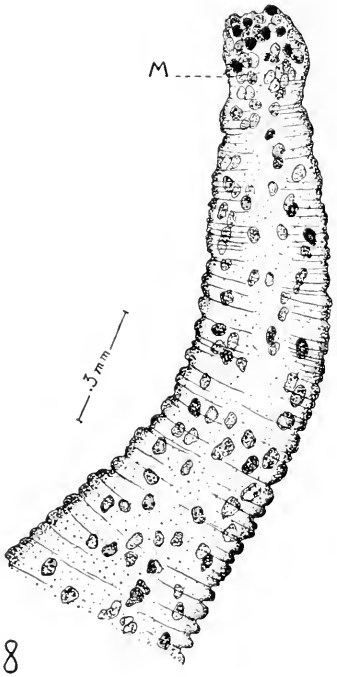
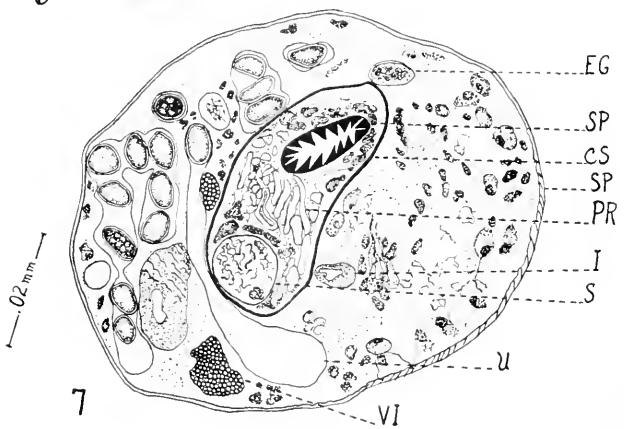


PLATE II







Description of Plate III

Fig. 6. Adult *Monorcheides cumingiae*.

Fig. 7. Transverse section of an adult *Monorcheides cumingiae*.

Fig. 8. Whole mount of a siphon of *Cumingia tellinoides* showing the distribution of metacercariae.

fed only infected *Cumingia* and after four weeks of feeding it was found to have an infection of over one hundred *Monorcheides cumingiae* in various stages of development. In all, four of the six fishes experimentally fed infected *Cumingia* were found to harbor *Monorcheides cumingiae* when examined four weeks after the initial feeding. In both the eels and the flounders, the parasites were found in the mucus of the anterior portion of the intestine. No ulceration of the intestine was observed. The controls were negative.

The adults are extremely small trematodes as shown by the following measurements based on five specimens, all of which contained eggs. The body length is 0.255–0.318, with an average of 0.30 mm.; the body width is 0.1–0.164, with an average of 0.142 mm. The cuticula is spined with the spines being somewhat more conspicuous on the anterior half of the body. The oral sucker is terminal or subterminal and measures from 0.0315–0.0415, with an average of 0.0367 mm. in length by 0.0348–0.0498, with an average of 0.0408 mm. in width. The ventral sucker measures from 0.0232–0.0332, with an average of 0.026 mm. in length by from 0.0291–0.0332, with an average of 0.032 mm. in width. The very short prepharynx (about 0.003–0.004 mm. in length) is followed by a pharynx that is on the average 0.0185 mm. in length by 0.022 mm. in width. The esophagus is approximately 0.0124 mm. long and it leads to the forking of the intestine which occurs about midway between the two suckers. The intestinal rami approach, but do not extend to, the posterior end of the body. The reproductive systems are well developed and both the cirrus and vagina are armed with relatively large triangular spines. The genital pore is located in the mid-ventral region just posterior to the forking of the intestine. The cirrus sac is elongate and extends from the genital pore to a short distance posterior to the ventral sucker. It encloses a seminal vesicle, numerous "prostate" cells, and a spined cirrus. The two testes are laterally situated about half-way between the ventral sucker and the posterior end of the body. They are composed of loosely arranged tissue. The single ovary is located just above the testis on the right side of the body. Its margin is irregularly lobate. No seminal receptacle was observed. The coils of the uterus may fill the posterior half of the body, particularly in the region between the testes. The uterus terminates anteriorly at approximately the middle of the median surface of the vagina. The vagina is relatively thick-walled, and is spined throughout its entire length. It is quite large, measuring approximately 0.0224 mm. wide by 0.0332 mm. long. It generally lies on the left side of the body. The spines arming the vagina are similar in shape and size to those found in the cirrus. The

eggs are amber-colored, oval in outline, operculate, and measure about 0.016 mm. long by 0.011 mm. wide. Mature eggs were removed from the uteri of several worms and were kept for over three weeks under conditions favorable for hatching but very little change took place in them. This suggests that the eggs must be eaten by the clam host before development will proceed.

The adult *Monorcheides cumingiae* differs from the other members of the genus in the following ways. The size of the body, suckers, pharynx, and eggs is smaller in *M. cumingiae* than in any other member of the genus. The shape of the vaginal and cirrus spines is triangular in *M. cumingiae* but in the other species, judging from figures, they are much more elongate. The spines of the cirrus of *M. cumingiae* are uniform in size while in *M. diplorchis* Odhner the spines on the median side are longer than those found on the lateral side of the cirrus. The vitellaria of *M. soldatovi* Issaitschikow extend from the posterior border of the pharynx to the level of the middle of the ovary, in *M. diplorchis* the yolk glands extend from the level of the forking of the intestine to a short distance posterior to the anterior margin of the ovary, while in *M. cumingiae* these glands may extend from the posterior margin of the ventral sucker to the anterior margin of the testes. *Monorcheides* (?) *petrowi* Layman probably does not belong to this genus at all because the ovary is posterior instead of anterior to the testes. The genus *Paramonorcheides* Yamaguti is closely related, if not synonymous, with the genus *Monorcheides*. The principal difference between the two seems to be a sac-shaped excretory bladder in the former and a Y-shaped one in the latter. Some specimens of *Monorcheides cumingiae* give the appearance of having a Y-shaped bladder due to the distention of the lower portions of the main collecting ducts. It is likely that Odhner believed that these enlarged collecting ducts were continuations and part of the bladder.

Nagano (1930), according to Yamaguti (1938), has shown that the larval form of *Asymphylodora tincae* is a tailless cercaria, or the so-called cercariaeum, lacking eyespots. It is apparent, therefore, that there are at least two larval types in the family. The larval form of the genus *Bivesicula* Yamaguti 1934 is evidently similar to the cercaria of *Monorcheides cumingiae* in the possession of eyespots since remnants of these structures are found in the adults.

Effect of Parasite on Clam

The effects of the parasite on the clam may be divided into two categories, first the damage done by the sporocysts when the clam serves as the first intermediate host, and second the injury caused by

the cercariae when they re-enter to become metacercariae or when the clam is serving as the second intermediate host. Of the two, the first undoubtedly is more extensive since most of the visceral region may become filled with sporocysts. The sporocysts seem to be more numerous along the wall of the gut and along the periphery of the body in lighter infections but in severe infections they fill the tissues between these two regions. The fact that the sporocysts first appear along the wall of the gut seems to support the evidence obtained from the unsuccessful efforts to hatch the eggs of this worm, that the eggs are eaten by the clam and hatch in the gut, infection taking place through the wall of the digestive tract. The normal tissue seems to be pushed aside and crowded upon itself until disintegration takes place. Here and there between sporocysts portions of host tissue may remain in an apparently normal condition. Just how this replacement of host tissue by the parasite occurs is unknown. Perhaps some of it is by an enzymatic dissolution of the host cells or perhaps it is by the compression of the host tissue by the parasite. If the latter is true, and there is some evidence to support this contention because here and there between the sporocysts distorted host cells can be found, the parasite tissue must be able to exert a greater growth pressure than the host tissue. The fact that host tissue may remain in certain regions entirely surrounded by sporocysts seems to indicate that the action is not an enzymatic one, or if it is, some of the host's cells are more resistant than others to this action.

The effects of the penetration of the cercariae are confined for the most part to those tissues of the clam that are in contact with the water, such as the siphons, mantle, foot, and gills. The siphon tissue, particularly that region next to the lumen and just below the muscular layer, is a favorite one for the penetration of the cercariae (see Fig. 1). Eighty metacercariae were counted in one longitudinal section of a siphon. The distal end of the siphon may become so filled with metacercariae that it presents a frayed appearance. Frequently the heavily infected siphon is autotomized. Although the incurrent siphon generally contains a larger number of metacercariae, the excurrent siphon may become rather heavily infected. Some encystment takes place in the mantle but probably because of its thinness it is incapable of supporting many metacercariae. The muscle of the foot of the clam becomes very heavily infected. Between ninety and one hundred metacercariae were observed in one longitudinal section of the foot. The metacercariae in this region seem to induce the formation of a deeply staining (with Delafield's hematoxylin) deposit which may be an attempt by the clam to protect itself. It, however, is not

a very effective protective device because recently encysted metacercariae were found in tissue that also contained older metacercariae. The mantle also may form small amounts of this deposit, in some cases even when there are no metacercariae present in that particular region. Therefore, it is likely that the deposition of this material is not a specific response to the action of the parasite but, nevertheless, the amount of the material deposited may be increased by the presence of the metacercariae. With the relatively large amount of surface exposed it seems rather remarkable that so few metacercariae are found in the visceral region. Perhaps the presence of sporocyst tissue may act as a barrier to the entrance of cercariae, or, it may be that the visceral tissue does not have sufficient rigidity for the penetration of cercariae for metacercariae are found in regions of the body where muscle fibers occur near the surface. The cercariae frequently penetrate the gill filaments forcing the thin membranes apart so that they surround the metacercariae. Some of the more heavily infected filaments are distorted considerably.

The damage done by the parasite to the invertebrate host is undoubtedly much more extensive than that received by the vertebrate host. However, in spite of the very extensive damage suffered by the clams they lived for several weeks, even without food except for the small amount that would be obtained from the daily changes of sea water.

Summary

1. The life cycle of *Monorcheides cumingiae* (Martin) involves the development of sporocysts, cercariae, and metacercariae in the marine bivalve, *Cumingia tellinoides*, and the development of the adult, under experimental conditions, in the flounder and eel.

2. Another clam, *Tellina tenera*, can serve as an alternative second intermediate host and on one occasion was found to act in the capacity of a first intermediate host.

3. Attempts at hatching the eggs of this trematode failed. Therefore it seems probable that the eggs must be eaten by the clam before further development will take place.

4. In spite of the extremely heavy infections, the clams survived for several weeks with no more food than would be obtained from the daily changes of sea water.

5. A free-swimming period is apparently necessary before the cercariae will encyst. This is an advantage to the parasite in increasing its range.

6. The cercariae encyst in large numbers in the siphons, particularly the incurrent, and foot. Metacercariae are found in smaller numbers in the gills, mantle, and, very rarely, in the visceral region.

7. There is a marked change in the staining reaction of the nuclei of recently encysted and older metacercariae. When both receive the same treatment, the nuclei of the young metacercariae take up little or no Delafield's hematoxylin while the nuclei of the older metacercariae are heavily stained.

8. Although the metacercariae seem to induce the surrounding clam tissues, particularly in the foot, to increase the deposition of a darkly staining material, this substance does not inhibit super infection.

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STUDIES ON THE EFFECTS OF EYESTALK REMOVAL UPON YOUNG CRAYFISH (CAMBARUS CLARKII GIRARD)

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In recent years it has come to be recognized as a result of the work of Megušar (1912), Abramowitz and Abramowitz (1938), Brown and Cunningham (1939), Hanström (1939), and Abramowitz and Abramowitz (1940) that removal of both eyestalks in decapod crustaceans causes the next moult to occur sooner than it would normally. Removal of eyestalks is eventually fatal, as Brown (1938) and Brown and Cunningham (1939) have shown for *Cambarus*, and Abramowitz and Abramowitz (1940) have shown for *Uca*. Brown and Cunningham give evidence, derived from sinusgland implantation, that the factor or factors related to moulting and viability originate in the sinusgland of the eyestalk.

In the above work the general method has been to operate on a number of animals at once, and then to compare the incidence of moulting in this group with that in an untreated group. This procedure gives no information as to the actual amount of shortening of the intermoult following operations, nor has sufficient evidence been presented to prove that the acceleration of moulting is a real shortening of the intermoult and not merely a speeding up of the intermoult in its later stages by the shock of eyestalk removal. The experiments to be described below were intended to clarify these questions by means of operations made after observed moults on animals whose history was known and whose moulting cycles could be followed individually.

To Dr. H. W. Rand, who kindly provided facilities for, and advice during, the preliminary experiments, and to Dr. J. H. Welsh, whose coöperation and criticisms have been invaluable, I wish to express my gratitude.

Experimental

In experiments carried out in 1938, young *Cambarus clarkii* were used, ranging in length from 17 to 24 mm. (rostrum to telson). These were kept in individual dishes at 9–14° C. and fed very lightly. Operations were performed two days after animals were observed to have moulted. Eyestalks were occluded by ligating them at the base with

fine hairs, while as a control operation antennae were similarly ligated and then removed with scissors. Other animals were left intact as controls.

It was found that removal of both eyestalks was always fatal. In the course of the eyestalk ligations discussed here, and some performed in another connection, 60 animals had both eyestalks tied off. Of these, 53 had died by the close of the experiment. Survival ranged from 1 to 38 days following the operation, averaging 17.1 ± 1.14 [1] days. If deaths within 10 days are disregarded the average survival was 19.7 ± 1.03 days. That this mortality is not due merely to operative injury is shown by the fact that only 2 deaths resulted from antennal removal among 30 animals.

Eyestalkless animals often moulted before dying, with intermoults shorter than in normal animals, but antennal removal had no significant effect upon the length of the following intermoult. Thirty-four crayfish had their eyestalks ligatured after the first observed moult. All died, but 14 moulted before dying, with intermoults averaging 15.9 ± 1.02 days, while in 29 normal animals the average was 28.9 ±1.65 days, and in 30 animals with antennae removed the average was 31.2 ± 2.28 days. This may indicate a significant shortening of intermoult by eyestalk removal, although the results are unsatisfactory because of the high percentage of deaths in the eyestalkless group.

Accordingly, the work was repeated in 1939, using a modified procedure. The essential feature of this second experiment was that operations were made only on animals in which one or more complete intermoults had been observed. Thus an intermoult following an operation could be compared with the preceding "normal" intermoult in the same animal. To hasten growth and moulting the animals were kept at room temperature and were fed liberally, the effort being made to supply as much food as they could eat. This had a marked effect upon survival that will be mentioned below.

Eyestalks were removed, with the animals held on ice, by pinching them off at the base with watchmaker's forceps. As a control operation the severe procedure of cutting off the retinal portion of the eyestalk with fine scissors was used. This caused an open wound with much bleeding. It was found advisable to perform the operations of eyestalk or retinal removal in two steps, taking off the second eyestalk or retina 12 hours after the first. Cautery was not employed.

[1] In this and following cases where standard errors are given, the errors are standard error of the mean, given by the formula $\frac{\text{standard deviation}}{\sqrt{\text{no. of variates}}}$.

The 122 *Cambarus clarkii* used ranged in length from 8 to 12 mm. (rostrum to telson) at the start of the 7-week observation period. Observations were made twice daily. When an animal had moulted twice (one intermoult), it was assigned to one of three experimental groups. The first animal was left as an intact control, the second had both eyestalks pinched off, the third had both retinas cut off. This series of assignments was repeated as moults occurred among the animals. Operations were started 24 hours after an animal was observed to have moulted. Crayfish showing excessively long intermoults were left out in the assignment to groups, but might be admitted later if the next intermoult were near the normal length. After intact controls had moulted a third time they were reassigned to whichever of the three experimental groups they happened to fall into. This resulted in the intact group being depleted of its more rapidly moulting members, which passed into and were recorded with, one of the two operational groups. As a result, the average intermoult for the remaining intact animals is abnormally long, and cannot be compared with the shorter pre-operational intermoults of the other two groups which will be discussed below.

No retinal removals were performed in the last 15 days of the experiment, while no eyestalks were removed in the last 11 days. There were thus more eyestalkless than retinaless animals recorded. A few eyestalkless animals were observed for 3 weeks after regular observations ceased in order to determine their survival times. The complete record of the intermoults observed in the two groups which had eyestalks and retinas removed is given in Tables I and II respectively. The intermoults which will be used to show the effects of eyestalk or retinal removal are the intermoults immediately before and following the operation.

In the group of crayfish whose eyestalks were removed, the intermoults immediately preceding operations averaged 12.13 ± 0.65 days, while the first intermoults following the operation averaged 8.10 ± 0.19 days (Table I). That this difference is significant is shown by the fact that the standard error of the difference of the means is 0.68 days, one-sixth the difference of the means. Of the 45 animals operated upon, only one died as a result of the operation, while a second has been disregarded in the calculations of mean intermoult and survival time because it is so obviously at variance with the rest. Twenty-two animals passed through a second intermoult after eyestalk removal. With two exceptions these second intermoults are as low as the first ones after the operations (Table I). Three animals moulted three times in the absence of their eyestalks. These third intermoults were

TABLE I.

Complete record of intermoults observed in the group which had both eyestalks removed, and the survival times of those animals which died naturally following eyestalk removal.

Pre-operational Intermoults (length in days)			Post-operational Intermoults (length in days)			Survival Time (days)
Earlier pre-operational intermoults		Last pre-operational intermoult	First post-operational intermoult	Second post-operational intermoult	Third post-operational intermoult	
		15.5	7.5	7.0		[30–31]
		10.0	8.5	8.5		18.0
		9.5	8.5	8.5		[25–30]
		11.0	8.5			Fixed
	15.5	14.0	8.0			23.0
	11.5	9.0	6.5			Fixed
		11.5	7.0	7.0		Fixed
	10.5	9.0	7.5			Fixed
		11.0	6.5			Fixed
		14.5	7.0			12.5
	16.5	16.5	7.5	8.0	11.5	26.5
		14.5	7.0			Fixed
	10.5	11.0	8.5	9.5		Fixed
		12.5	7.5			Fixed
		12.0	7.5	8.0	10.5	26.0
	[13.0]	[9.0]	[16.0]	[12.0]		[45]
		10.0	7.0	7.0		Fixed
	13.0	11.0	7.0	8.5		[25–30]
		13.0	7.0	7.5	11.0	26.5
		14.0	11.0			19.5
14.0	9.0	10.0	8.5	9.0		[22–27]
		13.5	7.5			Fixed
	14.0	12.0	9.5			9.5
		16.0	7.5	9.0		[16–21]
	14.0	11.5	8.0	9.5		Fixed
		13.0	Died			——
		13.5	12.0			12.0
12.0	8.0	11.0	7.0	[22.0]		28.5
	11.0	[20.0]	8.0	9.0		26.5
		11.0	7.0	8.0		22.5
		10.0	7.5			13.0
		11.0	8.5			Fixed
		16.0	8.5			Fixed
	11.0	11.5	8.0			Fixed
	13.5	11.5	7.5	7.5		[20–25]
	12.0	14.5	7.5	7.5		[22–27]
	14.0	13.0	11.0			11.0
		13.5	8.5			Fixed
	11.0	10.0	7.5	8.0		22.0
		15.0	10.0			10.0
		13.0	8.0	10.0		28.0
	9.5	9.0	8.0			Fixed
	10.5	10.0	10.0			17.0
		10.5	10.0	9.0		18.0
13.0	11.94 ± 1.16	12.13 ± 0.65	8.10 ± 0.19	8.30 ± 0.17	11.0	19.47 ± 1.48

TABLE II

Complete record of intermoults observed in the group which had both retinas removed.

Pre-operational Intermoults (length in days)		Post-operational Intermoults (length in days)		
Earlier pre-operational intermoult	Last pre-operational intermoult	First post-operational intermoult	Second post-operational intermoult	Third post-operational intermoult
	16.5	Died		
12.0	9.0	9.5		
13.0	7.5	7.5		
	13.5	17.5		
15.5	12.5	14.0		
	10.5	17.5		
	15.5	Died		
10.5	8.0	12.5		
	13.0	9.5	9.5	
	14.5	20.0		
15.5	10.5	17.5		
	11.5	16.0		
	10.5	11.5	13.5	
10.0	12.5	10.0	11.5	
	9.5	12.0	13.0	
	13.0	12.5	12.0	15.5
11.0	17.0	12.5		
13.0	13.0	14.0		
	13.0	Died		
11.5	8.0	13.0	9.5	
9.5	11.0	14.0		
11.5	12.0	14.5		
	14.0	16.5		
	16.0	9.5		
	12.5	12.5	11.0	
13.0	10.0	14.0	14.0	
12.0	14.5	11.5		
14.5	8.5	15.5		
11.5	11.0	21.0		
7.5	10.5	18.5		
	13.5	14.5		
	9.5	Died		
	9.5	10.5	9.0	
11.0	10.5	21.0		
11.91 ± 0.50	11.82 ± 1.04	14.02 ± 0.62	11.44 ± 0.58	15.5

longer than the preceding ones, but as eyestalkless animals eat a great deal, and become distinctly larger than normal animals of the same age, this increase is not surprising. The number of second and third intermoults of eyestalkless animals would have been greater had not

about a third of the animals been fixed for histological examination at the end of the first intermoult after eyestalk removal.

The effects of retinal removal are in contrast to those of eyestalk removal. With a few exceptions the post-operational intermoults are longer than the preceding ones (Table II). Intermoults in 30 retinaless crayfish average 14.02 ± 0.62 days, while the intermoults just before the operations average 11.82 ± 1.04 days. The increase in the length of intermoult after retinal removal is, however, of doubtful significance, since the difference between the means is only 1.8 times the standard error of the difference. In a few cases a shortened intermoult followed retinal removal. It is difficult to remove completely the retinal zone of the eyestalk without including some ganglionic material, leading one to suspect that in these cases the sinusgland may have been removed as well as the retina. The severity of retinal removal is shown by the fact that 4 deaths resulted from 34 operations. However, the remaining animals continued perfectly healthy, no other deaths occurring during the whole observation period. Retinaless animals did not attain the size of eyestalkless animals, a fact probably related to their slower rate of moulting. They tended to become quite dark in color, in contrast to the eyestalkless animals, which took on a pronounced reddish-brown color, becoming much paler after ecdysis.

Discussion

The results obtained show that eyestalk removal causes a shortening of the following intermoults in young crayfish. The mechanism suggested by Brown and Cunningham (1939) and Hanström (1939), that the eyestalks normally produce a hormone tending to delay or inhibit moulting, seems to be a reasonable explanation.

It has been shown that even severe injury does not shorten the intermoult, at least when the injury occurs early in the intermoult. Darby (1938) has stated that operative injury appears to hasten the next moult in *Crangon armillatus*. Possibly, however, his results may mean that the shock of injury late in the intermoult period causes a speeding up of moulting processes already well advanced in the animal, while injury early in the intermoult may have no such effect. It may be necessary to distinguish between the acceleration of moulting caused by eyestalk removal and that reported by Darby as caused by other types of injury.

The observation of Abramowitz and Abramowitz (1940) that *Uca* may occasionally moult more than once after eyestalk removal has been found to be true also for *Cambarus*, where three intermoults have been recorded after eyestalk removal. It seems beyond question that

the processes leading up to moulting can be initiated in the absence of the eyestalks, while "shock" effects are ruled out as a cause of the shortened intermoult.

A comparison of the survival time of eyestalkless animals in the second experiment with that of those in the first reveals a curious discrepancy. In 1938, with cold water (9°–14° C.) and rather scanty food, the average intermoult among eyestalkless animals was 15.9 days, the average survival 17.1, or at most 19.7 days. In 1939, with warm water (20°–22° C.) and unlimited food, the average intermoult in eyestalkless animals fell to 8.1 days, as might be expected, but survival averaged 19.5 days. Although not proved, it seems probable that the abundant food supply increased the expected survival time in 1939 over that in 1938. Otherwise survival in 1939 would have been shortened by warmth and favorable conditions to the same extent that intermoults were shortened. This observation, that in eyestalkless animals shortening of intermoult and time of survival are not necessarily parallel effects, suggests that the processes related to viability in the eyestalkless crayfish may be separated from those processes which underlie the more rapid moulting of such animals. However, no evidence so far presented is sufficient to show whether these processes are controlled by one substance from the eyestalks, or by two or more.

The fact that eyestalkless crayfish may moult successfully shows that the aid of the eyestalks is not indispensable to the physiological changes occurring at ecdysis, but that the moulting processes may be seriously interfered with by lack of the eyestalks is suggested by Abramowitz and Abramowitz (1940), who report that most of the deaths after eyestalk removal follow close upon a moult. This can be verified from the writer's second experiment. Twenty animals were maintained after eyestalk removal until they died. Table III shows that deaths fall into two main groups. The first is directly after the time of moulting, the second about 8 days later. Since the average

TABLE III

Survival time of eyestalkless crayfish after the last moult

Survival time in days	0*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Number of deaths	2	6	2	—	—	—	2	—	2	2	1	—	1	—	—	—	1	1

* Died in moult.

intermoult of eyestalkless animals is a little over 8 days, it seems probable that the second group of deaths is that of animals which died in an incipient moult, and suggests that the physiological changes associated

with moulting impose a severe strain which the animal fails to meet without the aid of some factor from its eyestalks. Presumably the weaker animals die before accomplishing ecdysis, while stronger ones may live long enough to moult, and still others may "recover" and pass through the next intermoult, to fail at some future moult. A few eyestalkless animals lived longer than 10 days after their final moults. This may possibly be because they were in such a weakened state that the next (incipient) moults were considerably delayed.

Summary

1. Removal of both eyestalks causes a shortening of the following intermoults in *Cambarus clarkii*. The removal of a moult-inhibiting substance produced in the eyestalks is accepted as a reasonable explanation.

2. The methods employed have been such as to show: (*a*) that the processes leading up to moulting can be initiated in the absence of the eyestalks; and (*b*) that the effect upon moulting is associated with the absence of the eyestalks, not with the shock of their removal.

3. Injury other than eyestalk removal does not shorten the intermoult when performed early in the intermoult.

4. Eyestalk removal always results in death, but the processes related to viability which are affected by eyestalk removal can be distinguished from the moulting processes which are affected by the same operation, although there is an apparent correlation between moulting and viability.

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EVIDENCE FOR THE PROTEIN NATURE OF THE SPERM AGGLUTININS OF THE KEYHOLE LIMPET AND THE SEA-URCHIN

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Chemical investigations on agglutinins (both naturally occurring and immune) for blood cells and bacteria have furnished strong evidence for the view that they are always of protein nature (see Marrack, 1938). It might be expected, then, that the sperm agglutinins present in the egg water of certain species of marine animals (see Lillie, 1919; Lillie and Just, 1924; Just, 1930; Tyler, 1940) would also show protein properties. This was not found to be the case by the earlier investigators. Glaser (1914) and Woodward (1918) applied a number of common protein tests to sea-urchin (*Arbacia*) egg water and obtained no reaction with the exception of a weak, partial xanthoproteic test. This does not, however, eliminate the possibility that the agglutinin is of protein nature, since it has often been shown that physiological responses can be evoked by solutions of proteins too dilute to give the ordinary tests. In fact, proteins can be detected in serological reactions at dilutions at which the color tests fail. For example, Uhlenhuth (1909) showed that a dilution of a protein as high as 1 to 100,000 will give a detectable reaction with anti-serum whereas neither the Biuret nor the Millon's reaction will exceed 1 to 10,000. Agglutination reactions are many times more sensitive than precipitin reactions (Zinsser, 1939; p. 246) and anaphylactic reactions still more sensitive.

In a preliminary note (Tyler and Fox, 1939) evidence that the sperm agglutinins of the keyhole limpet and of the sea-urchin are proteins has been reported. The present article presents the details of this evidence and further information on the properties and possible methods of purification of the agglutinins.

Bioassay

The agglutination reaction in the keyhole limpet, *Megathura crenulata*, has recently been described (Tyler, 1940*a*) in some detail. It was shown that the time at which agglutination of the sperm first becomes macroscopically visible increases with increasing dilution of the egg water (agglutinin), within certain limits and for a given sperm

suspension. This relation between concentration and agglutination time can be employed in a bioassay. For this purpose an arbitrary agglutination time is taken as a standard and the dilution of the test solution that gives the standard agglutination time is determined. The reciprocal of the dilution factor gives the agglutinin titer in arbitrary units. Since with concentrated solutions the agglutination time changes very much less than with dilute solutions, it is advisable to take as a standard a reaction obtained in a relatively dilute solution. We have usually taken as a standard a reaction that is first visible macroscopically in 30 seconds when equal volumes of the agglutinin solution and a 1 per cent sperm suspension are mixed. Solutions giving the standard reaction time are designated as having one unit concentration of agglutinin. The unit concentration selected is well above the weakest solution that gives a perceptible reaction. The latter end point, however, is not as easily determined. Sperm suspensions from different animals as well as suspensions of different ages and history from the same animal will, of course, show considerable variation in their reaction time even when employed in closely similar concentrations. The units have only approximate significance when comparisons of different series of tests are made. In most instances, however, the comparisons are made between control and test solutions simultaneously on samples of the same sperm suspensions. The concentration of agglutinin in the test solution is then given as a fraction or percentage of that in the control.

Other methods of bioassay that were tried with the keyhole limpet included determining the number and size of the clumps of agglutinated sperm and centrifuging in a hematocrit tube. These two methods did not prove as convenient nor as reproducible as the method of determining the time for agglutination to become macroscopically visible.

The agglutination reaction in the sea-urchin, *Strongylocentrotus purpuratus* (Loeb, 1914; Lillie, 1921), is quite similar to that in *Arbacia* (Lillie, 1913). The reaction occurs so rapidly even with dilute agglutinin solutions that it is not feasible to use the time at which it first becomes visible for bioassay. The method employed by Lillie (1914) is preferable. He showed that in the sea-urchin the agglutination reverses (i.e. the sperm disperse) within a short period of time ranging from a few seconds to a few minutes. The time at which reversal occurs decreases with decreasing concentration of the egg water. Lillie defined as a unit a solution of such concentration that the reaction reverses within three to five seconds, the observations being made under the microscope. For greater convenience we employed, as a standard, a reaction that reverses in one minute, the observations

being made macroscopically. A solution which, when mixed with an equal volume of a 1 per cent sperm suspension, gives a reaction that lasts one minute, is then designated as having one unit concentration of agglutinin.

Extraction

As Lillie first showed with *Arbacia* and *Nereis*, it is unnecessary to treat the eggs in any special manner in order to obtain agglutinin solutions. The sea water above the eggs becomes charged with the substance, the concentration increasing continuously with time. Allowing eggs to age in sea water is, however, not a very satisfactory method for obtaining solutions of high agglutinin titer. The eggs slowly disintegrate as they remain in sea water. This not only encourages bacterial growth and adds to the impurities but also liberates an anti-agglutinin (Lillie, 1914; Tyler, 1940*b*).

The method we have employed consists simply in dissolving the jelly layer surrounding the egg by means of acidified sea water. As was shown in the previous article, the agglutinin is either the jelly substance itself, or a component of it. The jelly slowly dissolves as the eggs age in ordinary sea water. In the sea-urchin, sea water acidified to pH 3.5 almost immediately dissolves the jelly layer without injuring the rest of the egg. A single extraction gives a solution containing practically all of the agglutinin that can be obtained from the eggs. A suspension of about 10^8 eggs in 100 cc. of acid sea water gives a solution of 32 units. Different preparations, however, vary widely in the yield.

In the keyhole limpet, the jelly layer does not dissolve as rapidly in acidified sea water as in the sea-urchin. There is, however, a rapid swelling and softening of the jelly and the eggs can be readily centrifuged out of their jelly hulls in the acid sea water. By this means agglutinin solutions of very high titer are rapidly obtained in the keyhole limpet too.

The agglutinins can also be rapidly extracted with isotonic NaCl acidified to pH 3.5. This was employed in some experiments in which it was desirable to eliminate certain of the sea water salts.

Rather weak or inactive preparations are obtained by such methods as freezing and thawing or extraction with distilled water which entail cytolysis of the eggs. This is due to the inactivation of the agglutinin by an anti-agglutinin (recently isolated by Tyler, 1940*b*) present within the eggs.

Extraction of the eggs by alcohol, acetone or ether failed to give active preparations. Also the agglutinin was not extractable from active precipitates (see below) or solutions by means of these solvents.

Dialysis

Lillie (1914) showed that the sperm agglutinin of *Arbacia* is non-dialyzable. We have found the same to be true for the sperm agglutinins of *Strongylocentrotus* and of *Megathura*. Concentrated solutions (approximately 20 to 30 units) of the agglutinins were placed in cellophane tubes or in collodion bags and dialyzed against approximately two to three times the volume of ordinary sea water with constant stirring. The dialysis was carried out in a cold room at 1° C. Samples were tested at regular intervals up to two weeks. The dialysate in all cases was completely inactive while the solutions within the tubes or bags retained practically their original activity during this period. Other samples that were dialyzed against running sea water likewise showed no loss in activity. It is evident, then, that the sperm agglutinins of the sea-urchin and of the keyhole limpet are substances of large molecular size.

Precipitation

It has been reported by Woodward (1918) that the sperm agglutinin of *Arbacia* partially precipitates when the egg water is saturated with $(NH_4)_2SO_4$. In *Strongylocentrotus* and *Megathura* we find that the agglutinins can be practically completely precipitated by the addition of $(NH_4)_2SO_4$.

When concentrated egg water of *Strongylocentrotus* or of *Megathura* is saturated with $(NH_4)_2SO_4$ a white flocculent precipitate slowly appears. The precipitation is usually complete within about 24 hours. The initial egg waters are usually opalescent in appearance. After removal of the precipitates the supernatants appear quite clear. Precipitates obtained from *Strongylocentrotus* and from *Megathura* egg waters were dissolved in sea water, dialyzed against running sea water to remove the $(NH_4)_2SO_4$, and adjusted to the original volumes. Agglutination tests with these solutions showed in all cases no significant differences from the original solutions. The supernatant solutions were also dialyzed and tested. With none of the latter was any sign of agglutination obtained. The following table gives the results of three such experiments with the keyhole limpet and two with the sea-urchin. The figures represent units concentration of agglutinin as defined above (p. 154).

	Megathura			*Strongylocentrotus*	
	A	*B*	*C*	*A*	*B*
Original egg water	16	8	6	32	24
Precipitate	12	10	8	32	20
Supernatant	0	0	0	0	0

It is evident that, in both *Megathura* and *Strongylocentrotus*, the agglutinin is completely salted out by saturation with ammonium sulfate.

An approximate determination was made, with *Megathura* egg water, of the lowest concentration of ammonium sulfate required for complete salting out of the agglutinin. To four 100-ml. portions of an approximately 10 unit agglutinin solution, made up in isotonic (0.55M) NaCl at pH 3, were added 30, 40, 50 and 55 grams of ammonium sulfate respectively. The salt dissolved completely in the first three and a small amount remained undissolved in the fourth. After standing 24 hours at room temperature the second, third and fourth flasks showed flocculent precipitates and clear supernatant solutions. The first flask showed no precipitate and the solution remained opalescent. The agglutinin titers of the precipitate and the supernatant in the second flask (40 grams) were determined after dialysis and adjustment to original volume. The precipitate gave practically the same agglutinin titer as the original solution; the supernatant showed no activity. Precipitation of the agglutinin is, then, complete in approximately three-fourths saturated ammonium sulfate.

A preliminary attempt at fractionation was made by slowly increasing the concentration of ammonium sulfate and removing the precipitate that first appears (at about 2/3 saturation) separately from that which comes out at higher saturation. Both fractions showed activity and, based on the relative centrifuge volumes of the precipitates, no marked difference in agglutinin titer was manifest.

Preliminary attempts at crystallization were also made with material prepared by dialysis, adsorption (see below), and repeated precipitation with 2/3 to 3/4 saturated ammonium sulfate by allowing the precipitation to take place in a dialyzing bag immersed in a solution of ammonium sulfate, the concentration of which was very slowly increased; also by slow evaporation of ammonium sulfate solutions of the agglutinin. In none of these were recognizable crystals obtained.

Adsorption

The agglutinins of both *Megathura* and *Strongylocentrotus* can be completely removed from solution by solid $CaCO_3$. For elution the $CaCO_3$ is dissolved by the addition of acid. This involves the disadvantage that some or all of the agglutinin may be inactivated by the acid (see below). To avoid this, it is necessary to employ a concentration of acid just sufficient to dissolve the $CaCO_3$ at a reasonable rate but not strong enough to inactivate the agglutinin. By use of sea water acidified to about pH 2.5 to 2.8 we have recovered approximately 25 to 50 per cent of the original agglutinin. Other methods of elution have not as yet been investigated.

The agglutinins are also adsorbed by Al_2O_3, charcoal and kaolin.

Color Tests

Several of the common color tests for proteins were applied to concentrates of sea-urchin and keyhole limpet agglutinins. The material was prepared by dialysis and ammonium sulfate precipitation of egg water obtained with the utmost care to avoid injury to the eggs themselves. In the case of the sea-urchin, it is quite feasible to prepare a concentrated egg water without any injury to the eggs and also to avoid or get rid of body fluid contamination. In the keyhole limpet this is more difficult unless one employs naturally shed eggs (see previous article). Definitely positive xanthoproteic, biuret and Millon's reactions were obtained with the agglutinin preparations from both sea-urchin and keyhole limpet. For the Millon's test it is necessary to dialyze against distilled water (washing the $(NH_4)_2SO_4$ precipitate on a membrane filter suffices) in order to remove salts, particularly chlorides, that interfere with the reaction. While the distilled water generally inactivates the agglutinin, this does not by any means invalidate the test as an indicator of the presence of protein. Attempts were also made to obtain sugar tests on portions of the material heated in concentrated HCl. These were all negative.

The failure of the earlier workers (Glaser, 1914; Woodward, 1918) to obtain positive protein tests with *Arbacia* egg water may very likely be due to their having worked with much weaker preparations. Positive reactions to these color tests do not, of course, prove that the agglutinin is of protein nature. They do, however, show the presence of proteins in the preparations. Since the method of obtaining the egg waters involves practically no destruction of the eggs themselves but simply dissolves the jelly layer, it is unlikely that there are very many different substances of high molecular weight present in the preparations. Previous evidence (Tyler, 1940*a*) had shown that the agglutinin is either the jelly substance itself or if the jelly is composed of more than one substance, that it is a component of it. If it were known that only a single high molecular weight substance were present in the preparations, then the color tests would be convincing evidence of the protein nature of the agglutinin. In place of such information other kinds of evidence have been obtained.

Action of Enzymes

Evidence for the protein or polypeptide nature of a substance may be obtained by digestion with proteolytic enzymes. The evidence is not very satisfactory when crude preparations of the proteinases are

employed since in such preparations there are generally other enzymes present. In recent years, however, a number of proteolytic enzymes have been crystallized (see Northrop, 1939), and these are, of course, eminently suitable for the tests. We have obtained, through the courtesy of Dr. J. H. Northrop, samples of crystalline trypsin and chymotrypsin and have examined their action on the sperm agglutinins of the keyhole limpet and the sea-urchin.

The *Strongylocentrotus* agglutinin is inactivated fairly rapidly by both trypsin and chymotrypsin. In four experiments that were run, inactivation was practically complete in less than 3 hours. The following figures give the agglutinin titers in one of the experiments in which solutions of trypsin, chymotrypsin and also steapsin (commercial) all adjusted to pH 8.0 were added to equal volumes of egg water, allowed to act at 20° C. and samples tested at the times indicated.

	2 min.	1 hour	2 hours	3 hours
Control	8	8	8	8
Saturated trypsin	8	6	3	0
1 per cent chymotrypsin	8	1/2	0	0
Saturated steapsin	8	8	6	6

The slight inactivation obtained with the commercial steapsin is perhaps to be attributed to small amounts of other enzymes present as impurities. The inactivation of the agglutinin by trypsin and by chymotrypsin occurs more rapidly than might be expected on the basis of the rate at which proteins are in general hydrolyzed by these enzymes. However, it is not necessary to assume that the substance must be split by the enzymes in order for inactivation to occur. In enzyme reactions there is generally considered to be an intermediate addition compound first formed. If, in the case of the agglutinin, the initial combination involved those groups that are concerned with its reaction with the sperm, there would be, in the presence of sufficient enzyme, a very rapid inactivation.

The *Megathura* agglutinin is much more slowly inactivated by solutions of the crystalline proteinases. In three experiments that were run, about seven days were required to reduce the titer to a point where no definite agglutination reaction is obtained. It is important, therefore, to have reasonably sterile conditions. This is relatively easy to do in the case of the keyhole limpet agglutinin since the solutions can withstand boiling for a considerable time (see below). The following figures (agglutinin concentration units—see bioassay section) give the course of the inactivation in one experiment run at pH 8 and 20° C.

	5 min.	1 day	2 days	3 days	5 days	7 days	8 days
Control	16	14	16	16	14	12	12
Saturated trypsin	16	14	12	12	10	1/2	0
1 per cent chymotrypsin	16	16	10	6	1/2	0	0

In this, as well as the other two experiments, the inactivation sets in slowly during the first few days and then proceeds more rapidly thereafter. Assuming that here, too, the initial combination of enzyme with substrate is quite rapid, it appears that this does not involve those groups on the agglutinin molecule which enable it to react with the sperm. The inactivation must then occur during the actual digestion of the agglutinin.

It would be desirable to determine whether or not there is a difference between the sea-urchin and the keyhole limpet agglutinins in regard to their manner of inactivation by these proteolytic enzymes. This could be tested by examining the digests for products of hydrolysis during the course of the inactivation. Due to limitations of material and to the desirability of using preparations that are of known purity for such purposes, the determinations have not as yet been made. One set of formol titrations has, however, been run on the digests (and controls) of the keyhole limpet agglutinin in the experiment listed above. The results showed that appreciable hydrolysis had occurred in the enzyme solutions. The determinations were made on aliquot samples removed on the tenth day. Solutions of trypsin and of chymotrypsin that had been kept under the same conditions as the digestion mixtures were added to samples of the control agglutinin solution at that time and the digestion mixtures were diluted correspondingly with sea water. Allowing for the controls the formol titrations gave 8×10^{-6} equivalents of $-COOH$ per ml. for the trypsin digest and 13×10^{-6} equivalents for the chymotrypsin digest.

Heat and pH Inactivation

The stability of solutions of both the keyhole limpet and the sea-urchin agglutinins varies with the temperature and the pH at which they are kept. Since the time for inactivation at a given temperature is a function of the pH of the solution, both of these variables may be considered together.

The sea-urchin agglutinin is fairly rapidly inactivated by heating the solutions. The following figures give the time required for half-inactivation and for practically complete inactivation of solutions of various pH at 100° C.

pH	2.50	3.1	4.2	6.3	7.3	8.2
Minutes for 50 per cent inactivation	.....	2–3	12–15	15–20	6–8	4–5
Minutes for 95–100 per cent inactivation	3	5–7	20–25	70–85	20–30	15–20

As the figures show, the agglutinin is most stable in the range of pH 4 to 7. At lower temperatures the time for inactivation increases, but the effect of pH remains substantially the same. Thus at 20° C. solutions at pH 4 retain their full activity for more than three days while pH 2 and pH 8 solutions are completely inactivated. At 0° C. solutions at pH 4 have been kept for over 6 months with no appreciable loss in activity.

When sea water solutions of the agglutinin are made alkaline (pH 9 and above), a precipitate of calcium and magnesium carbonates and hydroxides forms and this adsorbs the agglutinin. The agglutinin can be completely recovered by dissolving the precipitate in acid sea water. Due to the formation of the precipitate and adsorption of the agglutinin, sea water solutions could not be used for determining the rate of inactivation in the more alkaline range. Solutions in isotonic NaCl were employed for this purpose and these showed a continuous decrease in stability as the pH was raised.

The keyhole limpet agglutinin is considerably more stable in solution than is that of the sea-urchin. The following figures give the time required for half-inactivation and for nearly complete inactivation of solutions of various pH at 100° C.

pH	1.2	2.0	3.2	5.0	8.2	9.2	11.0
Hours for 50 per cent inactivation	1/6	1–1½	24–36	21–30	5–9	4–6	1/30
Hours for 95–100 per cent inactivation	1/2	3–3½	60–90	44–90	14–18	14–18	1/6

The determinations at pH 9.2 and 11.0 were made on solutions of the agglutinin in isotonic NaCl, since, as in the case of the sea-urchin, the precipitate that forms upon the addition of alkali to sea water adsorbs the agglutinin. Here again the agglutinin can be completely recovered by dissolving the precipitate. The pH range of maximum stability of the keyhole limpet agglutinin is roughly the same as that of the sea-urchin. It is, however, considerably more stable, being able to withstand boiling for more than 24 hours with only a 50 per cent loss in activity. At lower temperatures the stability varies in the

same manner with pH. Solutions of pH 2.5 to 5.5 kept at room temperature remain fully active for more than a week while solutions at pH 1 and pH 11 are completely inactivated in less than a day. At 0° C. the solutions of pH 2.5 to 5.5 have been kept more than 6 months with no loss of activity.

The inactivation of agglutinin solutions by heat may be considered to be due to a denaturation of the active substance. With concentrated solutions of both the keyhole limpet and the sea-urchin agglutinins a precipitate forms upon inactivation by heating. It is possible, however, to inactivate completely the agglutinins without the appearance of a precipitate or coagulum. This occurs generally with dilute solutions. The failure of a coagulum to appear upon inactivation of dilute solutions does not, however, exclude the possibility that the effect is due to denaturation of the substance. Denaturation is generally assumed to involve more than one step (see Mirsky, 1938), the final one being coagulation.

Dry Weights and Nitrogen Content

The concentration of organic matter was determined in agglutinin solutions of keyhole limpet and of sea-urchin prepared with special care to avoid injury to the eggs and contamination with body fluids, etc. Samples of the solutions were dialyzed against distilled water of known solid content, evaporated to small volume by boiling and dried at 80° C. to constant weight. Micro-Kjeldahl nitrogen determinations were then made on the dried material. The results were as follows:

		Megathura	*Strongylocentrotus*
Titer of Solutions		32 units	16 units
Organic solid content	range	0.10–0.14%	0.028–0.031%
	average	0.11%	0.03%
Nitrogen content	range	2.8–5.2%	3.8–5.9%
	average	4.6%	5.2%

The low content of organic solid matter may perhaps account for the failure of the earlier workers to obtain positive protein tests on sea-urchin agglutinin. The nitrogen content (about 5 per cent) is evidently too low for a pure protein. However, we do not as yet know how much inactive material may be present. The present values may be taken then merely as showing the presence of a definitely detectable quantity of nitrogen which is consistent with the other evidence that the agglutinin is of protein nature.

The amount of solid matter contributed per egg may be estimated roughly for the sea-urchin from the fact that a 16 unit solution is ob-

tained by extracting 5×10^7 eggs in 100 cc. of acid sea water. Since 100 cc. of the solution yields 30 mg. of organic solid matter, a single egg contributed 6×10^{-7} mg. In the absence of information as to the amount of inactive material present, this value may be taken as representing a maximum for the quantity of agglutinin obtainable per egg. The volume of an egg of *S. purpuratus* is 2.6×10^{-7} cc. and, assuming a density of 1.04, the wet weight would be 2.7×10^{-4} mg. From the figures of Leitch (1934), the dry weight would be about one-fifth of the wet weight or 5×10^{-5} mg., and roughly three-fourths of this is protein. The quantity of agglutinin obtained from a single egg would then correspond at most to 1 per cent of the dry weight of the egg.

Discussion

From the method of extraction, the non-dialyzability, precipitation with $(NH_4)_2SO_4$, the color tests, the heat- and pH-lability, the insolubility in alcohol and ether, the presence of nitrogen and particularly the inactivation by means of purified proteinases, one may conclude that the sperm-agglutinin both of the sea-urchin and of the keyhole limpet is either protein or closely associated therewith. This is consistent with the results on other naturally occurring agglutinins as well as immune agglutinins (and antibodies in general) that have been investigated (see Marrack, 1938; Heidelberger, 1938; Landsteiner, 1936; Zinsser, 1939). It is also in line with the specificity of the reaction.

The other active substances that have been isolated from sperm and eggs: namely, the anti-agglutinins, egg agglutinins and egg membrane lysin (Tyler, 1939, 1940*b*), also appear to be of protein nature. It would seem, then, that there is some justification for the analogies which Lillie drew between the fertilization reaction and immunological reactions. However, considerably more work will be necessary in both fields before we can determine to what extent immunological principles may be used to interpret the fertilization reactions.

The sperm agglutinins of the two animals investigated differ markedly, as the results show, in certain properties. That of the keyhole limpet is considerably more resistant to inactivation by heat (and pH change) and by proteolytic enzymes than is that of the sea-urchin. The situation is, however, not unusual. Marrack (1938, p. 50) reports that the heat stability of agglutinins (and antibodies in general) is very variable. Even in the same sera considerable differences in lability between different agglutinins have been found. Different antibodies also differ in regard to their resistance to the action of proteolytic enzymes (Marrack, p. 52). The destruction proceeds in

many cases quite slowly and in some instances use has been made of the relative resistance to digestion in attempts to purify antibodies (see Zinsser, p. 172).

The difference in stability between keyhole limpet and sea-urchin agglutinins correlates with differences in the nature of the reaction in these two forms. It has been previously shown (Tyler, 1940*a*) that the sperm agglutination reaction in the keyhole limpet persists considerably longer than does the reaction in the sea-urchin. Corresponding to the difference in duration of the reaction, a difference was found in the time at which precipitation occurs when the active principle (anti-agglutinin) from sperm is added to agglutinin solutions. In both species inactivation of the agglutinin occurs immediately after addition of the anti-agglutinin but while precipitation occurs within a few minutes in the sea-urchin it is many hours later in the keyhole limpet. These differences are not necessarily attributable entirely to the agglutinins, since the properties of the sperm and the sperm extracts cannot be assumed to be the same in the two species. Nevertheless, it is of interest to note that if greater stability is assumed to mean also slower reactivity on the part of the substance, then the differences in the properties of the keyhole limpet and sea-urchin agglutinins reported here afford a reasonable interpretation of the difference in the agglutination reaction in these two forms. In other words, the reactivity of the keyhole limpet substance may be considered to be lower than that of the sea-urchin.

Summary

Some of the chemical and physical properties of the sperm agglutinin (fertilizin) of the keyhole limpet *Megathura crenulata* and of the sea-urchin *Strongylocentrotus purpuratus* were investigated. In both species the agglutinins were found to be non-dialyzable. They precipitate completely without loss of activity in nearly saturated ammonium sulfate. They are adsorbed by $CaCO_3$, Al_2O_3, charcoal and kaolin. The agglutinins are insoluble in alcohol and ether. Active concentrates give, contrary to the findings of earlier investigators, the common color tests for proteins and are found to contain nitrogen. Solutions of crystallized proteinases (trypsin and chymotrypsin) inactivate the agglutinins. They are also inactivated by heat as a function of pH. From the evidence it is concluded that in both species the agglutinating principle is either protein or very closely associated with protein.

The keyhole limpet agglutinin is much more resistant than is that of the sea-urchin to inactivation by heat and by proteolytic enzymes.

It is suggested that the difference between the two species in the duration of the agglutination reaction may be related to the difference in stability of the respective agglutinins.

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A COMPARISON OF THE DEVELOPMENT OF NUCLEATE AND NON-NUCLEATE EGGS OF ARBACIA PUNCTULATA

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A further study has been made of the development of the non-nucleate half-eggs or parthenogenetic merogones of *Arbacia punctulata*, especially in comparison with similar nucleate half-eggs or fertilized merogones. A study has also been made of the cytological details as shown in sections of fixed material, and of the reaction with the Feulgen technique. In order to have a better understanding of the development of the parthenogenetic and fertilized merogones (red halves), a further study has been made of the fertilized nucleate half-eggs (white halves) in comparison with similar whole eggs both normal (uncentrifuged) and centrifuged. Differences in the development of these eggs and half-eggs are caused not only by differences in nuclear content, whether both ♂ and ♀ nuclei are present, or only one, or none at all, but also by the shape of the eggs and differences in cytoplasmic content caused by the centrifugal force.

This comparative study is presented in a series of photographs arranged especially for comparison, and the reader is requested to study the plates which are almost self-explanatory.

Material and Methods (Plate I)

The normal *Arbacia* egg when centrifuged (3 minutes at 10,000 × g) stratifies, elongates, becomes dumb-bell shape and then breaks into halves; these halves with further centrifuging elongate and break into quarters. This is shown in Plate I (Photographs 1–11), and has been described in previous papers (1932, 1936). The important facts are that the nucleus always goes intact to the light pole under the oil

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EXPLANATION OF PLATES

The photographs are all of living eggs, except those in Plate VIII, which are of sections. The photographs of Plates I to VI (except 64) are all as nearly as possible of the same magnification, approximately 250×. The photographs of Plate VII and 64 are also all to the same magnification, approximately 60×. The photographs of Plate VIII were taken with an oil immersion lens and magnified approximately 400× as presented, except 130 and 131, which are magnified about twice that amount. The times given under each photograph refer to the time after fertilization or activation at 23° C.

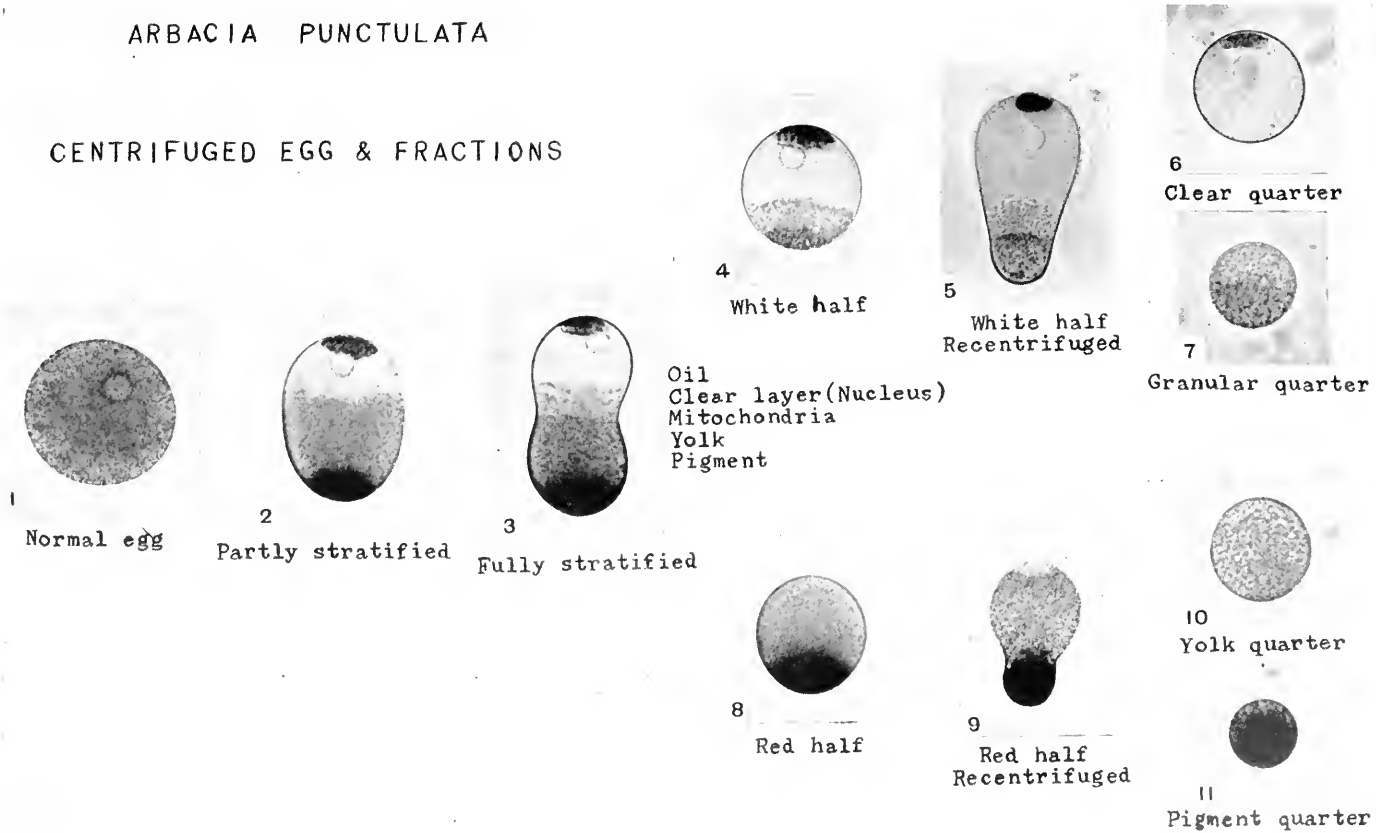

PLATE I

Centrifuged egg and fractions

The actual sizes (diameters) are as follows: whole egg 74 μ, white half 62 μ, clear quarter 56 μ, granular quarter 40 μ, red half 56 μ, yolk quarter 52 μ, pigment quarter 32 μ. (See 1932 paper.)

cap, and that the halves and quarters are uniform in size, but differ from each other in cytoplasmic content as well as in size. All of the halves and quarters can be fertilized; all will throw off a fertilization membrane and at least begin development. When the elongate whole egg and the elongate halves are fertilized, they become "set" and retain their shape. If left for an hour or so in sea water, unfertilized, they become spherical and the granules partially redistribute. The white and red halves develop much better if allowed to stand an hour or so before fertilizing them.

The whole eggs, both normal (uncentrifuged) and centrifuged, will develop parthenogenetically if treated for 20 minutes with a hypertonic salt solution made by boiling sea water to half its volume or by adding 30 grams of NaCl per liter of sea water. The white (nucleate) halves develop parthenogenetically with practically the same treatment, and the red (non-nucleate) halves also develop to a certain stage. It is of interest that a *hypo*tonic solution will cause parthenogenesis as well as a *hyper*tonic solution; the immersion of the sea-urchin egg for about a minute in distilled water will cause activation (Schücking, 1903); but the eggs of *Arbacia punctulata* develop only to the amphiaster of the first cleavage and only rarely cleave with this treatment.

Study of Development

Whole Egg, Centrifuged then Fertilized (Plate II, 12–15)

The elongate centrifuged whole egg, when fertilized immediately, develops as shown in Photographs 12–15. The chief points of interest are that the cytoplasmic materials remain partially segregated and the first cleavage plane comes across the short axis in a rather definite position, so that the first two cells are unequal. The pigmented cells are throughout the cleavages, generally larger than the unpigmented. Micromeres have not been observed. In spite of the peculiar cleavages,

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Plate II

Centrifuged and normal egg

Photographs 12–15. Centrifuged egg, fertilized immediately.
Photographs 16–32. Normal (uncentrifuged) egg.
Photograph 17. Monaster stage.
Photograph 18. "Streak" stage.
Photograph 19. Nuclear membrane just broken.
Photograph 20. Amphiaster.
Photograph 22. Immediately after first cleavage; cells well separated.
Photograph 23. Just before second cleavage; cells close together.
Photograph 26. Micromere stage; 12 cells.
Photograph 28. Blurring caused by swimming of blastula inside membrane.
Photograph 29. Hatching from fertilization membrane.
Photograph 30. Late blastula; note cilia. The animal was narcotized.
Photograph 31. Gastrulation has begun. Note cilia.
Photograph 32. Well-formed pluteus. Notice lattice-like skeleton in arms.

CENTRIFUGED & NORMAL EGG, FERTILIZED

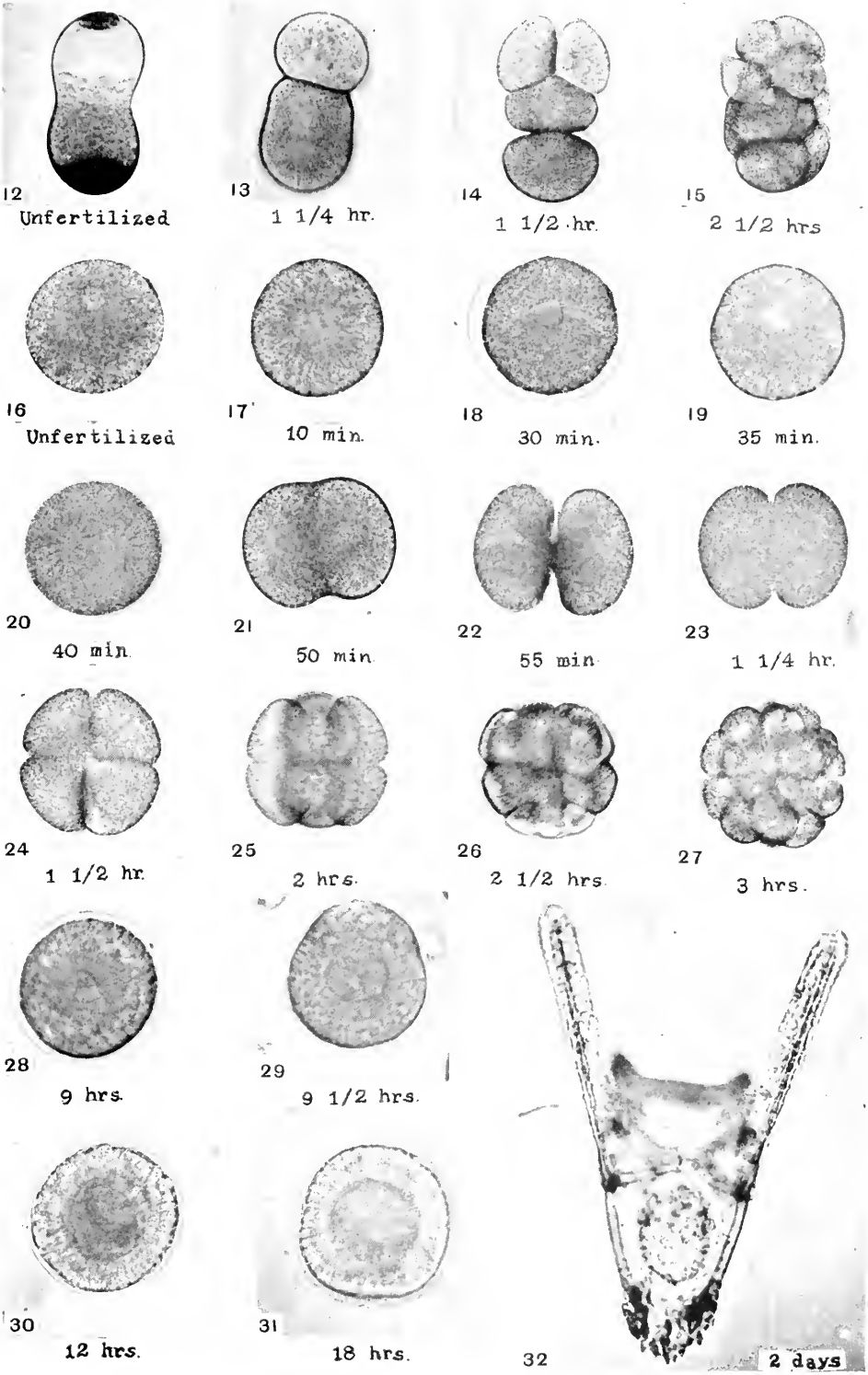


PLATE II

which result in slipper-shaped blastulae, plutei are formed which are normal in every respect except for the concentration of pigment in certain areas. Usually the pigment is near the oral end, but it may be in any position, as originally described by Lyon (1907).

Normal (Uncentrifuged) Whole Egg, Fertilized (Plate II, 16–32)

Some stages in the development of the normal *Arbacia* egg are shown in Photographs 16–32. The especial characteristics are that the first three cleavages are equal and that at the next cleavage micromeres are formed, small colorless cells, giving a definite 12-cell stage (Photograph 26). A peculiarity following the first cleavage is that the two cells are at first widely separated (Photograph 22) and later become pressed together, probably owing to the formation of the next mitotic figure (Photograph 23). The blastulae hatch out from the fertilization membrane when having some 500–600 cells (computed from photographs),[1] and they have only a small blastocoel. The skeleton of the *Arbacia* (*punctulata* and *pustulosa*) pluteus is the lattice type like that of *Sphaerechinus granularis* and unlike the simple rods of *Paracentrotus lividus*, *Parechinus microtuberculatus* and *Strongylocentrotus dröbachiensis*.[2]

Whole Egg, Parthenogenetic

Both the normal (uncentrifuged) and the elongate (centrifuged) egg will develop parthenogenetically similarly to the fertilized egg but more slowly. Plutei have been obtained in both cases.

[1] This agrees fairly well with Morgan's (1895 *a*, *b*) estimate of 500–526 cells for *Sphaerechinus granularis* and 1,000 for *Echinus microtuberculatus*, and MacBride's (1914) figure of 808 for *Echinus esculentus*, at the time of hatching. The number of cells computed for *Arbacia* would represent nine cleavages (2^9).

[2] A very interesting study of these skeletons with regard to systematic relationships of the adults is given by v. Ubisch (1932).

PLATE III

White half, fertilized

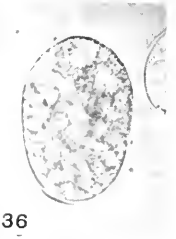
Photographs 33–36. Elongate white half.
Photographs 37–53. White half after standing an hour after centrifuging.
Photograph 38. Monaster stage. Monaster does not show well, owing to lack of heavy granules.
Photograph 39. "Streak" stage. Streak does not show so well as in normal whole egg, but the enlarged nucleus is plainer.
Photograph 40. Nuclear membrane just broken.
Photograph 41. Amphiaster.
Photograph 43. Immediately after first cleavage; cells well separated. Cf. 22.
Photograph 44. Before second cleavage; cells close together. Cf. 23.
Photograph 49. Blurring caused by blastula swimming inside membrane.
Photograph 50. Hatching from fertilization membrane.
Photograph 51. Late blastula.
Photograph 52. Gastrula.
Photograph 53. Well-formed pluteus. Note lattice-like skeleton in arms. Cf. 32.

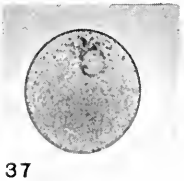
WHITE HALF, FERTILIZED

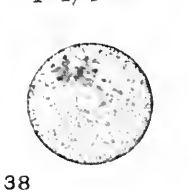

33
Unfertilized

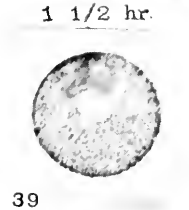

34
1 1/4 hr.

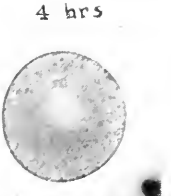

35
1 1/2 hr.

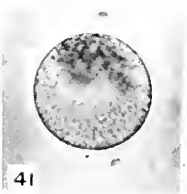

36
4 hrs

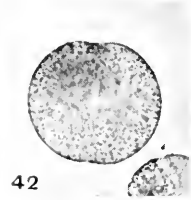

37
Unfertilized

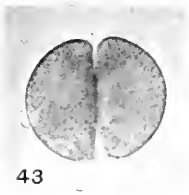

38
10 min.

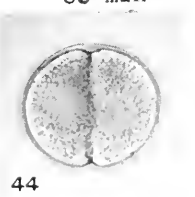

39
30 min.

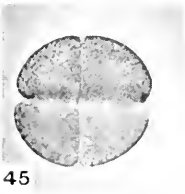

40
35 min

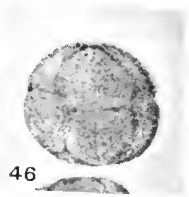

41
40 min.

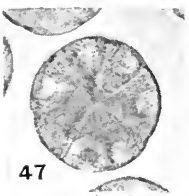

42
50 min

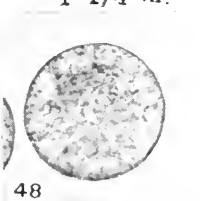

43
55 min.

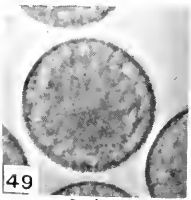

44
1 1/4 hr.

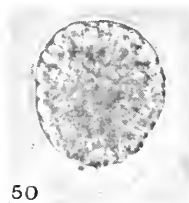

45
1 1/2 hrs.

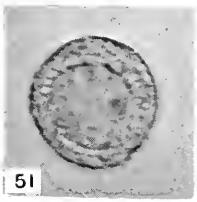

46
2 hrs.

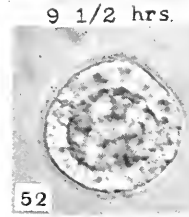

47
2 1/2 hrs.

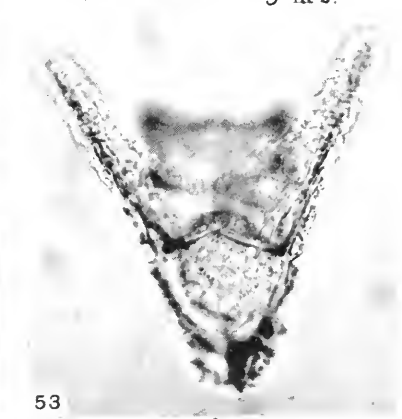

48
5 hrs.


49
9 hrs


50
9 1/2 hrs.


51
15 hrs.


52
24 hrs.


53
4 days

White Half-egg (Plates III and IV)

The development of the white half-egg, made elongate by further centrifuging (20–30 minutes at 10,000 × g) and immediately fertilized, is shown in Photographs 33–36. It will be noted that the first cleavage plane goes across the short axis and divides the egg unequally (Photograph 34); it is usually in a position corresponding to that in the elongate whole egg (Photograph 13). The subsequent cleavages are similar in the two cases, and slipper-shaped white blastulae are formed (Photograph 36).

The development of the white half which has been allowed to stand for an hour or so till the granules are more evenly distributed, and then fertilized, is shown in Photographs 37–53. This half-egg lacks all the red pigment and most of the yolk granules and yet it cleaves and develops quite like the normal whole egg. After the first cleavage, the two cells are at first well separated and then come close together (Photographs 43, 44) as in the normal egg. Micromeres have not been observed, but they would be difficult to be sure of on account of the small size of the cells, and the lack of color contrast. Plutei have been raised, normal in every respect except for size and lack of pigment (Photograph 53). After a few days, pigment granules appear. The skeleton is of the lattice form typical of the pluteus from the whole egg.

Well-stratified spherical white halves as they occur immediately after having been centrifuged off from the whole egg (Photograph 54), will also develop when fertilized. The first cleavage plane comes in usually through the oil cap (Photograph 55), as it does in whole eggs which have been well stratified but not elongated; this was observed

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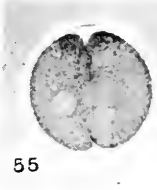
PLATE IV

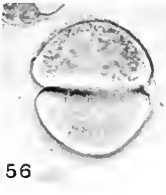
White half fertilized, continued

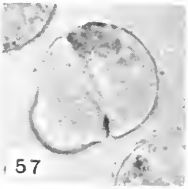
Photograph 54. White half, immediately after removal from centrifuge.
Photograph 55. First cleavage through oil cap.
Photograph 56. First cleavage parallel with stratification.
Photograph 57. First cleavage diagonal.
Photographs 58, 59. White halves, together with whole eggs and red halves, fertilized, to show comparative development in same lot of eggs. Higher magnification of lot similar to 116.
Photographs 60–64. Abnormal development of white halves. Cf. Photographs 11–14 of 1940 paper.
Photograph 60. "Dauerblastula."
Photograph 61. Blastula with primitive triradiate spicule.
Photograph 62. Blastula with abnormal skeleton.
Photograph 63. Abnormal pluteus without arms.
Photograph 64. A group of abnormal plutei from one batch, together with one normal whole pluteus from the same batch of eggs.

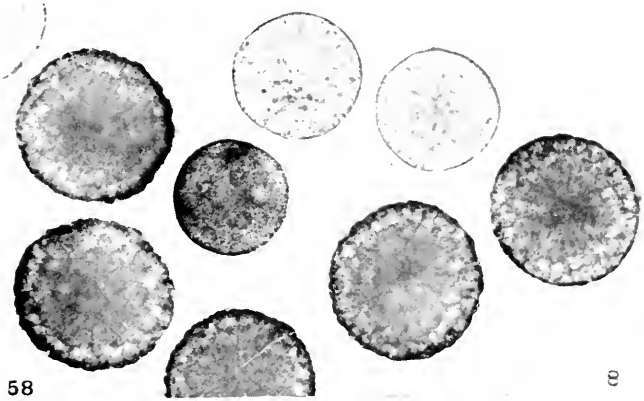
WHITE HALF, FERTILIZED

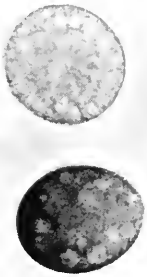

54

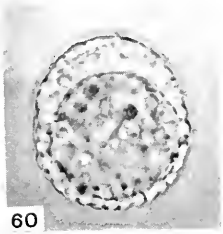

55

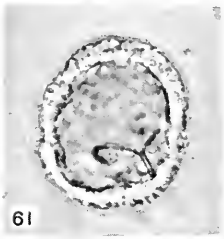

56

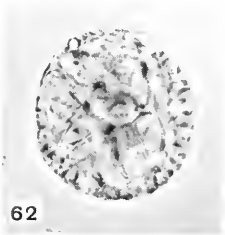

57

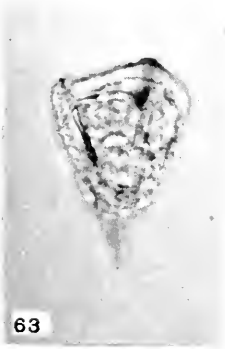

58


59
8 hrs


60
3 days


61
3 days


62
3 days


63
3 days


64
3 days

PLATE IV

for the whole egg in the first centrifuging experiments of Lyon (1907). He also observed that the first cleavage plane may come in, less frequently, parallel with the stratification, or at an angle. This is true also of the spherical white halves (Photographs 56–57).

Although many of the white halves develop into normal plutei, some develop abnormally. Among the abnormalities are permanent blastulae ("Dauerblastulae"), blastulae with primitive triradiate spicule or with imperfect skeleton, and abnormal plutei with skeleton and no arms (Photographs 60–64). In a recent paper (1940), it was shown that white halves obtained by centrifuging after fertilization may develop into abnormal blastulae and plutei quite similar to these, but in this case no normal plutei occur. Compare photographs 60–63 of this paper with 11–14 of the previous paper (1940). There seems to be no constant percentage of normal development in any one batch of white half-eggs, but certain whole batches develop much better than others, owing probably to better experimental conditions. In some batches, all are abnormal (Photograph 64; one normal pluteus from whole egg present for comparison).

The white half-egg will also develop parthenogenetically, and give rise to a white pluteus similar to that obtained from the fertilized white half.

Red Half-egg, Fertilized; Fertilized Merogone (Plate V)

The development of an elongate red half-egg, obtained by centrifuging a little longer (5 minutes at 10,000 × g) and in a slightly denser sugar solution, and immediately fertilized, is shown in Photographs 65–68. This egg has only the ♂ nucleus. The first cleavage plane is across the short axis and divides the egg unequally (Photograph 66), as in the elongate whole egg and white half. The following cleavages are likewise similar (Photographs 67, 68).

The development of the spherical red half is given in Photographs 69–88, and has been previously described (1932). The fertilization membrane and ectoplasmic layer are thicker than in the white halves. Fairly regular cleavages may take place, but with the pigmented cells usually larger (Photographs 73–77). A blastula, with small blastocoel is formed, and this emerges from the fertilization membrane in quite typical fashion and becomes free-swimming (Photographs 78, 79). Complete development into plutei is rare, and only a few normal or almost normal plutei have been obtained (Photograph 80).[3] In some cases, the fertilization membrane breaks during cleavage, and the cleavage cells spread out (Photographs 81–84). Also, the cleavage planes are apt to be omitted after nuclear division, so that multi-

[3] A number of absolutely perfect small plutei with lattice-like skeletons have recently (July 29) been obtained from fertilized red halves.

nucleate forms are common, or forms in which a few cleavage planes occur (usually in the lighter portion) and many nuclei in the uncleaved portion (Photographs 84–88). It seems likely that the great amount of heavy granular material interferes with the cleavage planes, and this may be responsible for the difficulty in raising these eggs to full development.

Red Half-egg, Parthenogenetic; Parthenogenetic Merogone (Plate VI)

The red half-eggs, though having no nucleus, can be activated artificially, by means of hypertonic sea water (see under "Material and Methods"), and they develop quite like the fertilized red halves, to a certain stage. They develop best if activated just after centrifuging, even if elongate. This may be due to the fact that the surface membrane is stretched and thinner and thus perhaps more permeable, so that the surface changes take place more readily. The development of the elongate egg is given in Photographs 89–92, and is similar to that of the elongate red half-egg, fertilized (Cf. Photographs 65–68).

The development of the spherical red half is shown in Photographs 93–112. The fertilization membrane and ectoplasmic layer are thick (Photograph 94) as in the fertilized red half. It will be noticed that a clear sphere is present a little later (Photograph 95), resembling the nucleus in the fertilized merogone; whether there is a definite membrane around it, is difficult to determine, though there seems to be a phase boundary. The monaster stage (Photograph 96) is common and striking, though the monaster is, in fertilized eggs, associated with the male nucleus which of course here is absent. Amphiasters are frequently seen and the cleavage plane may come in between the two asters in typical fashion (Photograph 97). The first cleavage plane may divide the egg equally, in any relation to the stratification, though more usually it is parallel with the stratification (Photographs 97, 98). Successive cleavages may be fairly regular, and a many-celled blastula formed (Photographs 99–101) exactly as in the fertilized red half (Cf. Photographs 73–77). This emerges in typical fashion from the fertilization membrane (Photograph 102), but has never developed into a pluteus. Some 500 cells have been counted in the cellular blastulae (Photograph 103), which is approximately the number of cells in a normal blastula at hatching (See p. 170, and footnote 1). There seems to be no blastocoel. The blastulae do not swim actively, though they move or are moved slightly. If they have cilia, they are short and irregularly distributed. Some of these organisms have lived for four weeks, and were still viable; they did not increase in size but were rather, smaller, and the pigment disappeared. (Photograph 104).

Just as in the fertilized merogones, the fertilization membrane frequently breaks during cleavage, and the cells become loosely arranged (Photographs 105–108; cf. 81–84). Also cleavage planes may be omitted, and multi-astral forms occur (Photographs 109–111), similar to the multi-nucleate forms of the fertilized merogone (Cf. Photographs 85–88). In both types, some of the cleavage planes come in and some are omitted, especially in the pigment portion (Photograph 108, right egg; cf. 84). In some batches of red halves, multi-astral forms occur spontaneously soon after activation. These are probably similar to the "artificial astrospheres" of Morgan (1896, 1899, 1900), and the "cytasters" of Wilson (1901) in parthenogenetic whole sea urchin eggs. In some batches there occur many blastulae in which very small clear spheres are observed, sometimes associated with the asters (Photographs 109, 111, 112). These certainly resemble small nuclei, and the similarity of these supposedly non-nucleate red halves to the nucleate (fertilized) ones is indeed striking (Cf. Photograph 112 with 88). These spheres may be re-formed nuclei without chromatin; the chromatin could hardly appear *de novo*, and no chromatin could be detected in stained preparations.

Many substances have been added to the sea water in an effort to obtain further development of the parthenogenetic merogones beyond the blastula. The substances were added in varying amounts before, after and during centrifugation, before and after activation. It was thought that possibly the substances might penetrate better while the

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PLATE V

Red half, fertilized (fertilized merogone)

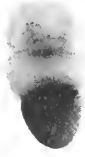
Photographs 65–68. Elongate red half.
Photographs 69–88. Spherical red half.
Photograph 70. Soon after fertilization, to show fertilization membrane and thick ectoplasmic layer.
Photograph 71. Male nucleus.
Photograph 72. Monaster.
Photograph 73. Amphiaster. Cell division will come in perpendicular to stratification.
Photograph 74. Two-cell stage. Division has been parallel with stratification.
Photographs 73–77. Regular cleavages with cell division.
Photograph 78. Hatching from fertilization membrane.
Photograph 79. Free-swimming blastula.
Photograph 80. Almost normal pluteus.
Photographs 81–84. Less regular cleavages, with fertilization membrane ruptured so that cells are more scattered.
Photograph 84, right. Egg in which cell divisions have come in in light part, but not in pigmented part, though nuclear division has taken place.
Photographs 85–88. Nuclear division without cytoplasmic division. Notice that the nuclei are not uniform in size in any one egg. Many small nuclei are present in Photograph 88.

RED HALF, FERTILIZED (FERTILIZED MEROGONE)

65 Unfertilized

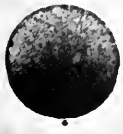
66 2 hrs

67 3 hrs

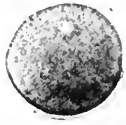


68 4 hrs.

69 Unfertilized



70 10 min.

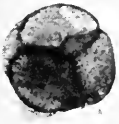


71 20 min.

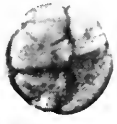
72 30 min.

73 1 1/2 hr.

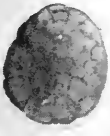
74 2 hrs.



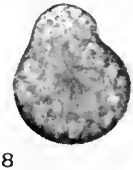
75 3 hrs.



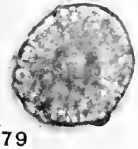
76 4 hrs.



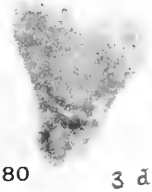
77 8 hrs.



78 11 hrs.

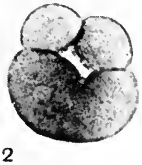


79 20 hrs



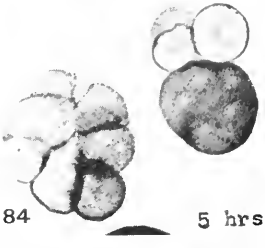
80 3 days

81 3 hrs

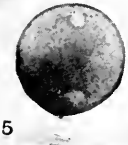


82 3 hrs.

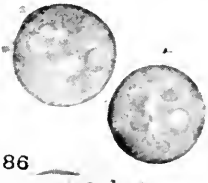
83 4 hrs.



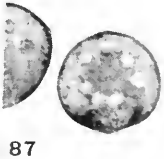
84 5 hrs



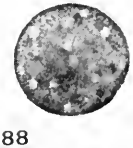
85 4 hrs.



86 6 hrs.



87 7 hrs.



88 9 hrs.

PLATE V

membrane was stretched in centrifuging. Though some batches of eggs seemed to develop better than the controls in certain solutions, the results were never reproducible. Among the substances tried were materials associated with the nucleus, hormones, vitamines and dyes, as follows:—killed *Arbacia* sperm, living frog nuclei macerated, thymus nucleic acid, yeast nucleic acid, adenine, guanine, uracil, tobacco mozaic virus, *Megatherium* phage, adrenalin, pituitary, theelin, ascorbic acid, auxin, methylene blue, rhodamine, and also glutathione and leukotaxin. Variation of temperature, of concentration of the sea water, and different parthenogenetic agents were also tried. Preliminary experiments have been carried out of injecting some of these substances into the egg, but the technical difficulties are great.

Comparison of Shape, Rate and Size (Plate VII)

From the preceding paragraphs, it will be seen that the position of the cleavage plane differs with the shape of the egg. In spherical eggs, the first cleavage plane divides the egg equally. In elongate eggs, it passes usually through the short axis, parallel with the stratification, and divides the egg unequally. This is irrespective of the specific materials in the egg or half-egg, whether mitochondria, yolk or pigment. The position of the cleavage plane in elongate eggs seems to be determined rather by the relative consistency of the layers; it comes in where there are some granules, but not too many, and

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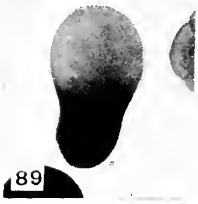
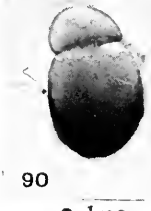
PLATE VI

Red half, parthenogenetic (parthenogenetic merogone)

Photographs 89–92. Elongate red half. Cf. 65–68.
Photographs 93–112. Spherical red half. Cf. 69–88.
Photograph 94. Soon after activation, to show fertilization membrane and thick ectoplasmic layer.
Photograph 95. Clear sphere simulating a nucleus. Cf. 71.
Photograph 96. Monaster.
Photograph 97. Amphiaster. Division will be perpendicular to stratification.
Photograph 98. Two-cell stage. Division has been parallel with stratification.
Photographs 97–101. Regular cleavages with cell division.
Photograph 101. Fine blastula with many cells. Cf. 77.
Photograph 102. Hatching from fertilization membrane. Cf. 78.
Photograph 103. Many-celled late blastula.
Photograph 104. Non-cellular parthenogenetic merogone, 4 weeks old.
Photographs 105–108. Cells somewhat scattered owing to rupture of fertilization membrane. Cleavages less regular and often asynchronous.
Photograph 106. Perfect 4-cell stage without membrane. Note asters.
Photograph 108, right. Egg in which cell divisions have come in in light part, but not in pigmented part. Cf. 84.
Photographs 109–111. Multi-astral eggs, with several small spheres associated with the asters. Cf. 85–87.
Photograph 112. Many small spheres resembling nuclei. Cf. 88.

RED HALF, PARTHENOGENETIC (PARTHENOGENETIC MEROGONE)

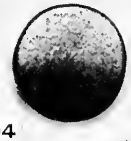

89
Unfertilized

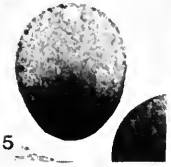

90
3 hrs


91
4 hrs.


92
18 hrs.


93
Unfertilized

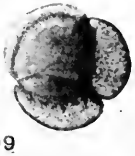

94
10 min.


95
1 hr.


96
1 1/4 hr.


97
2 1/2 hrs.

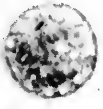

98
3 hrs.

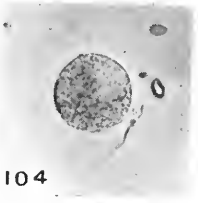

99
4 1/2 hrs.


100
5 hrs.


101
27 hrs.


102
28 hrs.

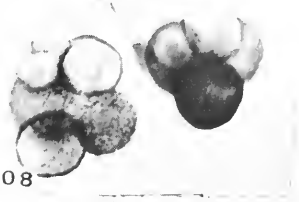

103
30 hrs.


104
4 wks.

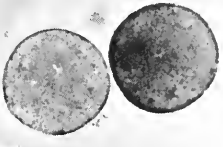

105
4 hrs.

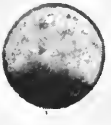

106
4 1/2 hrs.


107
6 hrs.


108
7 hrs.


109
3 hrs.


110
6 hrs.


111
10 hrs.

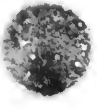

112
10 hrs.

PLATE VI

usually not in the very narrowest part. The cleavage pattern does not, however, seem to affect final development.

The fertilized white halves cleave at the same rate as the whole eggs, if anything a little in advance (Photographs 113–116, 58, 59), and they hatch from the fertilization membrane at the same time. They are slower to differentiate; they are still blastulae when the whole eggs have become plutei (Photograph 117), and they become plutei the following day, but they are smaller than normal ones (Photograph 118). They are at first colorless, but acquire pigment later on. The parthenogenetic eggs, both whole ones and white halves, are slower in cleavage and development than the fertilized ones.

The fertilized red halves (fertilized merogones) cleave more slowly than the normal wholes and white halves. After 4 hours (Photograph 119), they are at about the same stage as the others at 2 hours (cf. Photograph 115). They hatch later, and differentiate still more slowly than the white halves. They are still blastulae after two days (Photograph 120), and become plutei on the following day (Photograph 121).

PLATE VII

Comparative

Photograph 113. Group of whole eggs, white and red halves to show comparative sizes.

Photograph 114. Similar group an hour after fertilization. The whole eggs and white halves are in the 2-cell stage, the red halves still uncleaved.

Photograph 115. Similar group a half hour later. The whole eggs and white halves are in the 2- and 4-cell stage, the red halves still uncleaved. The white halves are slightly in advance of the whole eggs.

Photograph 116. Similar group 7 hours after fertilization. The whole eggs and white halves are blastulae, the red halves much behind. The inset at right is a group of the whole eggs printed more lightly to show cleavage planes. A group similar to this, more highly magnified, is shown in Photographs 58, 59.

Photograph 117. The day after fertilization, the white halves are still spherical blastulae, the whole eggs are plutei.

Photograph 118. The following day, the white halves have become plutei; the small pluteus is from a white half, the others from whole eggs of the same age.

Photograph 119. A mixed group, printed lightly to show red halves, in 2- and 4-cell stages after 4 hours, similar to wholes and whites after 1½ hours. Cf. 115.

Photograph 120. The red half still a blastula after two days. A whole pluteus of same age alongside.

Photograph 121. The red half has become a pluteus the following day. A normal pluteus of the same age is alongside.

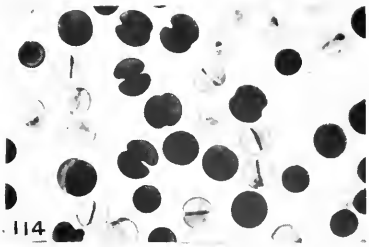
Photograph 122. A pure culture of parthenogenetic merogones, 4 hours after activation. Note the large numbers of cleaved eggs, in 2-, 3-, and 4-cell stages. The normal fertilized egg is many-celled at this time; cf. 27.

Photograph 123. A parthenogenetic merogone after three days, still a spherical blastula, of same size as previously. A normal pluteus of the same age is alongside.

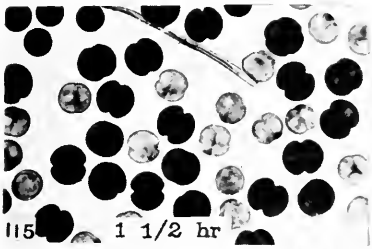
COMPARATIVE


113

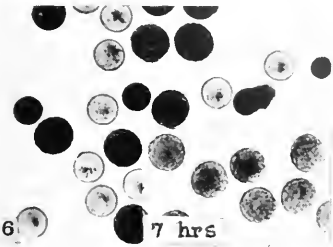
Unfertilized


114

1 hr


115

1 1/2 hr

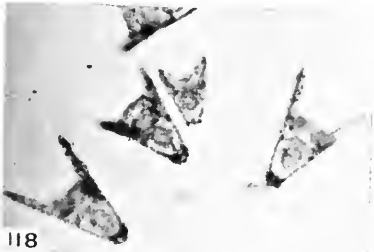

116

7 hrs

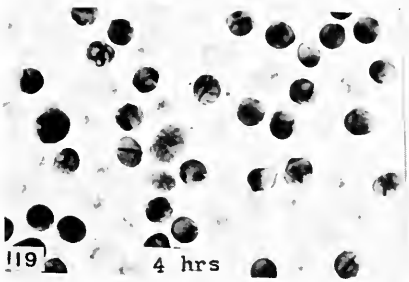
White halves, red halves & wholes


117

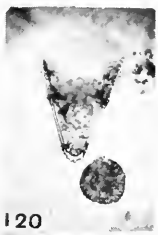
1 day


118

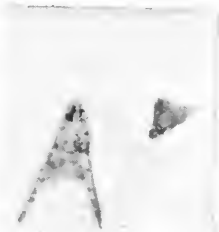
White half & wholes 2 days


119

4 hrs

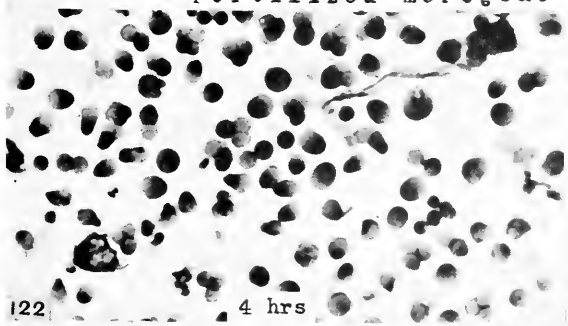

120

2 days

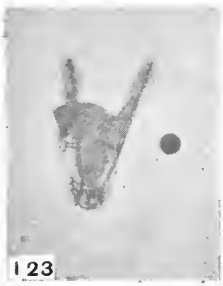

121

3 days

Fertilized merogone & whole


122

4 hrs


123

3 days

Parthenogenetic merogone & whole

The parthenogenetic merogones develop still more slowly. First cleavage takes place in about three hours, whereas it is two hours for the fertilized merogone, and 50 minutes for the white halves and wholes. In 4 hours they are a little behind the fertilized red halves (Photograph 122). The parthenogenetic merogones usually hatch only after 24 hours, whereas the whole eggs hatch in 9½ hours. And as stated above, they do not differentiate into plutei (Photograph 123).

As a general comparison of rate of cleavage, the eggs with two nuclei cleave more rapidly than those with one, whether parthenogenetic whole eggs or fertilized merogones. Those with one nucleus cleave more rapidly than those without any. The slow rate of cleavage of the clear quarters which have two nuclei is an exception (Harvey, 1932). Twice the amount of nuclear material in comparison with the cytoplasm, as occurs in the fertilized white halves, does not appreciably affect the rate of cleavage. But as we have seen, these halves differentiate, i.e. become plutei, more slowly. Possibly the lack of the full quota of cytoplasm may be responsible for the delay in differentiation.

The process of cell division and cell multiplication can go on with two nuclei, or one nucleus or none at all. Up to the present time, the parthenogenetic merogone has not gastrulated nor acquired a skeleton; practically no differentiation has taken place. And it may be that nuclear material is necessary for differentiation.

No attempt has been made to compare accurately the nuclear size of the half and whole eggs. A glance at Photographs 59, 74, 78 and 86 will show how variable is the size in a single egg. Also the nucleus in a normal egg changes in size before first cleavage from 11.5 μ to 16 μ. The nuclei in the clearer portions of the eggs are in general larger than in the pigmented portions in the same size cells.

No special granules seem to be necessary for development since both halves of the eggs, containing certain granules and lacking others, can develop into plutei. The red pigment seems to interfere with cell division but not with final development.

It should be stated that the times given for the various stages are by no means invariable. They depend upon the time of year as well as upon temperature and experimental conditions. This is especially true of the red halves which are very variable, both fertilized and parthenogenetic. Based on eight years of experience with the material, the times given are an average for development, as accurately as could be obtained, for a temperature of 23° C. at the height of the season.

Study of Stained Sections (Plate VIII)

Stratified Eggs (Photographs 124–127)

The eggs were fixed immediately after centrifuging, in Bouin's fluid, sectioned and stained with iron hematoxylin, and some were counter-stained with eosin and orange G. The clear layer of the whole egg (Photograph 124), which is optically empty in the living egg, stains blue, and is granular; this is apparently the protoplasmic ground substance, not moved by the centrifugal force used. This was described also in the early paper of Lyon (1907). The yolk stains orange with a rose tinge and the pigment orange. The mitochondria can sometimes be distinguished as a darker bluish band between the protoplasm and the yolk. The oil cap does not show; it is probably dissolved in the fixing or clearing fluid. The white half stains, of course, like the upper part of the whole egg (Photograph 125). The red half (Photograph 126) usually shows a blue cap of protoplasm, though this is obscured in the living egg by granules. In the red half further centrifuged till it is elongate, this cap of protoplasm formed by the further packing of the granules toward the heavy end, is larger (Photograph 127), and it is often seen in the living egg as a clear layer (Photograph 9).

Eggs Fertilized, Then Centrifuged (Photographs 128, 129)

When the eggs are fertilized and then centrifuged, the nucleus is always at the light pole, as in unfertilized eggs. At the stage after the nucleus has enlarged and the chromosomes have begun to form, just before the breaking of the nuclear membrane, the chromatin material is thrown to the heavy end of the nucleus as a dense mass (Photograph 128). This does not happen in the unfertilized egg (Photograph 124). The nucleolus of the immature egg is, however, thrown down to the centrifugal end of the germinal vesicle. If such fertilized eggs as mentioned above are left (living) in sea water after centrifuging, the material redistributes within 7 minutes, and normal spindle formation and cleavage follow.

When the fertilized eggs are centrifuged after the spindle has formed, it goes intact (with the forces used) to the light pole (Photograph 129) as found by Spooner (1911). There is no chromatin material left behind either before or after the nuclear wall has broken, as it would be perfectly visible in these stained preparations. There is no possibility, therefore, that the red halves contain chromatin material from the nucleus.

Chromosome Numbers (*Photographs 130, 131*)

The diploid chromosomes in the cleavage figures of fertilized eggs are small and crowded, and are very difficult to count with certainty, as other investigators have also found. The number is between 32 and 38 (Photograph 130). Tennent (1912) gives "about 40" for *Arbacia punctulata*, and Morgan (1927, p. 627) gives 38, quoting Wilson's data of 36–38. The number in the first cleavage of the parthenogenetic whole egg is approximately half the diploid. In Photograph 131, there are 16, but one or two may be missing.

The Mitotic Figure (*Photographs 132–140*)

In the early cleavages of the normal *Arbacia* egg, the spindle and asters are beautifully formed; the asters have rather thin rays. In Photographs 132–134 are given prophase, metaphase and anaphase figures. The mitotic figures of the fertilized white halves are similar to the normal egg. The fertilized merogone has also a spindle and

PLATE VIII

Sections

(These photographs have been touched up to bring out details more clearly)

Photographs 124–127. Unfertilized eggs fixed in Bouin immediately after centrifuging, sectioned and stained in hematoxylin, eosin and orange G.

Photograph 124. Whole egg, showing protoplasm above, mitochondrial band, yolk and pigment. The oil cap does not show. Cf. living egg, Photograph 3.

Photograph 125. White half. Cf. living egg, Photograph 4.

Photograph 126. Red half; a protoplasmic layer is at the centripetal pole, not visible in living egg, Photograph 8.

Photograph 127. Red half, centrifuged further. Protoplasmic layer is greater; this is visible in a similar living egg due to greater packing of granules. Cf. Photograph 9.

Photograph 128. Fertilized egg centrifuged just before breakdown of nuclear membrane, showing chromatin material thrown down to heavy pole of nucleus. In the living egg, not centrifuged, this would be Photograph 18.

Photograph 129. Fertilized egg centrifuged at the metaphase, soon before cleavage. The spindle is intact at the centripetal pole. This is the stage shown in the living uncentrifuged egg in Photograph 20.

Photograph 130. Diploid group of chromosomes; 32–38.

Photograph 131. Haploid group from parthenogenetic whole egg; 16, one or two may be missing.

Photographs 132–134. Mitotic figure of normal fertilized egg. Prophase (132), metaphase (133) and anaphase (134). Forty to 45 minutes after fertilization.

Photographs 135–137. Mitotic figure of fertilized red half (fertilized merogone). Prophase (135), metaphase (136) and anaphase (137). Note slender spindle, and thick astral rays. One to 2 hours after fertilization.

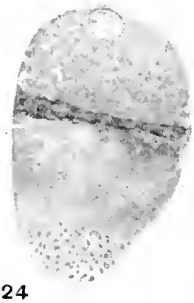
Photographs 138–140. Mitotic (?) figures of the parthenogenetic merogone.

Photograph 138. Monaster. Note thick rays. One and ¼ hour after activation.

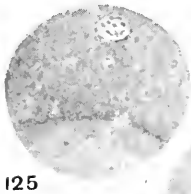
Photograph 139. Asters present in pairs in 2-cell stage. Note that those in the pigmented part have thicker rays than those in yolk. Four hours after activation.

Photograph 140. Two eggs overlapping, one in amphiaster stage before first cleavage, one in three-cell stage, each cell with a pair of asters. Six hours after activation.

SECTIONS



124
Whole egg



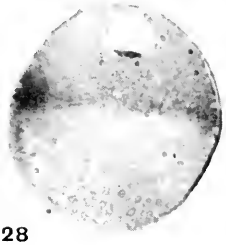
125
White half

Stratified

126
Red half

127
Red half

130
Diploid



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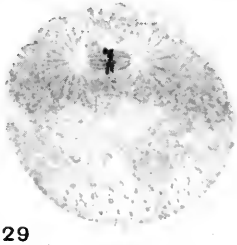
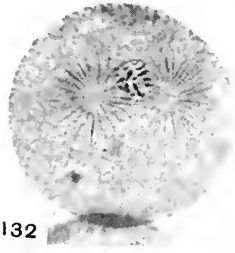


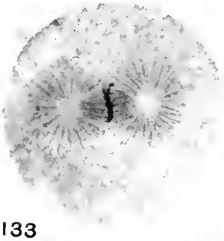
129

Fertilized, then centrifuged

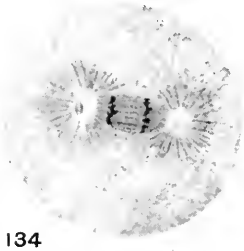
131
Haploid



132

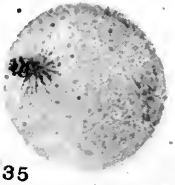


133

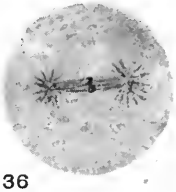


134

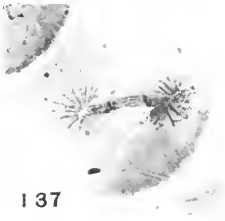
Normal whole egg



135

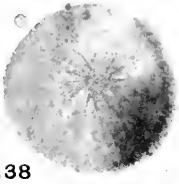


136

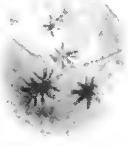


137

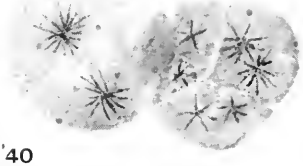
Fertilized merogone



138



139



140

Parthenogenetic merogone

asters; the spindle is much thinner, often curved, and the rays of the asters are shorter and thicker; they are thicker in the pigmented than in the yolk portion. In Photographs 135–137 are given prophase, metaphase and telophase figures of the fertilized merogone. The parthenogenetic merogone has well-formed asters but no spindle; the rays of the asters are even heavier and thicker than in the fertilized merogone and thicker in the pigment area than in the yolk (Photographs 138–140). A large monaster is characteristic of the parthenogenetic merogone (Photograph 138), appearing in the same sequence of events as the monaster which develops in connection with the sperm in fertilized eggs. Later, two asters appear in the same sequence as the amphiaster in the fertilized eggs; the rays may approach each other toward the center, but there is never a well-formed spindle. In later cleavages, the asters may be found in pairs, also without spindles. In Photograph 40, there is one egg with an amphiaster and another egg, overlapping, with three cells, in each of which is a pair of asters. The original of the last photograph was sketched by four different investigators independently, and the sketches all agreed essentially. There is no question that asters are present, often in pairs, in the parthenogenetic merogones, but no spindle and no chromosomes. There is no special granule or centriole in any of the asters in the sea urchin egg.

Feulgen Reaction

The Feulgen reaction, which is specific for chromatin, is negative for the parthenogenetic merogones. Professor Jean Brachet very kindly helped me with the technique and examination of the eggs. The parthenogenetic merogones showed no red-staining material, whereas the fertilized merogones, prepared in the same way at the same time, showed it very clearly. There is apparently no chromatin material in the parthenogenetic merogones, at least in the early cleavages.

Summary

1. A comparative study has been made for *Arbacia punctulata*, especially by means of photographs, of the development of the normal whole egg, the white half, the red half fertilized (fertilized merogone) and the red half parthenogenetic (parthenogenetic merogone), all of these both spherical and elongate. The comparative rate of development is also given.

2. Development of the parthenogenetic merogones is not improved by applying various substances to the outside, such as nuclear compounds, hormones and vitamines.

3. A study of the stratification of the centrifuged egg and its halves as seen in prepared sections has been made.

4. Sections of eggs fertilized and then centrifuged show that at a certain stage, the chromatin material is thrown to the centrifugal pole of the nucleus, and that the spindle goes to the centripetal pole intact.

5. Cytological details have been studied, in sections, of the division figure of the normal egg, of the red half fertilized, and of the parthenogenetic merogone. During cleavage stages, the parthenogenetic merogone has well-formed asters, often in pairs, but no spindle and no chromosomes.

6. The Feulgen reaction is negative for the parthenogenetic merogones; there is no chromatin material.

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SOME EFFECTS OF COLCHICINE UPON THE FIRST CLEAVAGE IN ARBACIA PUNCTULATA[1]

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The pioneer work of Hertwig and Hertwig (1887), Boveri (1897), Wilson (1902) and others on the effects of various environmental conditions upon the division mechanism of cells has stimulated numerous workers to further study the effects of a wide variety of agents such as anaesthetics, poisons, injury, hypertonic and hypotonic solutions, centrifuging, mechanical agitation and various types of radiation upon this interesting process.

The recent advent of the use of colchicine (a narcotic alkaloid) to produce polyploidy in cells has led to numerous publications, mainly by cytogeneticists, on this general subject. However, few studies have been made in an effort to analyze the general effects of colchicine on the cell as a whole and particularly from the point of view of the mechanics of cell division. It was with these problems in mind that we undertook the present investigation.

Material and Methods

In preliminary experimentation it was found that 0.0002 molar concentration of colchicine in sea water when applied 10 minutes after fertilization was effective in preventing the formation of mitotic figures or cleavage furrows in the eggs of *Arbacia punctulata*. Furthermore, this solution was not lethal to the eggs as determined by their ability to undergo subsequent cleavage after being removed from the colchicine solution to sea water.

Experiments were therefore designed to determine at what stage or stages in the mitotic cycle the eggs could be prevented from cleaving by treatment with this concentration of colchicine. Accordingly, eggs were placed in the colchicine solution at two-minute intervals over a period of one hour after fertilization, at which time some of the eggs were examined for mitotic figures and cleavage furrows; some were fixed for a detailed study of the asters, spindle and chromosomes, and some were allowed to remain in the solution for 6 hours or longer to determine whether or not the cleavage mechanism was completely blocked or possibly only temporarily delayed by the treatment.

[1] Aided by grant from the Rockefeller Foundation for work on Cellular Biology.

To determine the effects of the colchicine upon the consistency ("viscosity") of the eggs, experiments were performed as follows: eggs were placed in the colchicine solution 10 minutes after fertilization and let stand 10 minutes. They were then centrifuged, together with controls, for the same time and at a speed just sufficient to produce a slight derangement but no extensive stratification of the control eggs. It is well known that the viscosity of the eggs varies considerably at different stages in the mitotic cycle. Therefore, untreated eggs of the same lot which were fertilized 10 minutes later than the experimental series were centrifuged for the same time and at the same speed. This was done in order to obviate the possibility that any difference in consistency between the experimental and control eggs might be due to a further development of the controls because of the possible blocking of the experimental ones by the colchicine solution.

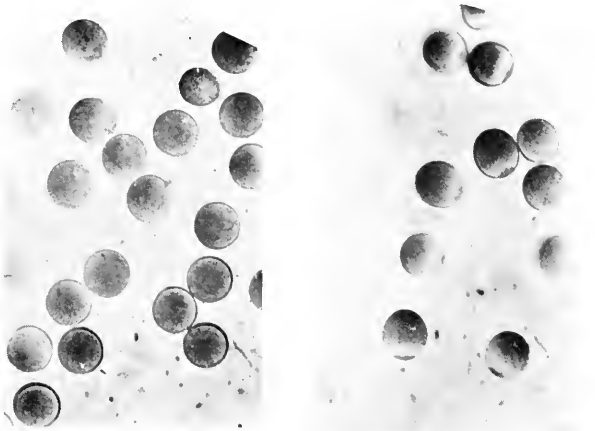

FIG. 1. Control eggs (left) and colchicine-treated eggs (right). Both were centrifuged 20 minutes after fertilization for the same time and speed. A comparison of the stratification indicates that a lowering of the viscosity has occurred in the colchicine-treated ones.

In each experiment only eggs from a single female and sperm from a single male were used. The eggs were kept in finger bowls at room temperature with the water varying from 22° to 24.4° C. Controls were run with each experiment.

Observations

All experiments in which the stratification of control and colchicine-treated eggs was compared after centrifuging showed the experimental eggs to have a lower viscosity as indicated by a more marked stratification of certain of their cytoplasmic components. The hyaline zone was only barely indicated in the controls, whereas it was well developed in the colchicine-treated eggs (Fig. 1). It is to be pointed out, however,

that the cortex of the experimental eggs did not in all instances show a complete displacement of their pigment granules, thus indicating a higher viscosity for the cortex than exists in the sub-cortical regions of the eggs. That this is true for the normally dividing eggs seems well established (Brown, 1934; Marsland, 1939). Higher centrifugal forces which produced definite hyaline areas in the control eggs also caused a derangement and some displacement of the pigment granules in the cortex of the colchicine-treated eggs, whereas in the cortex of the control eggs similar effects were not so striking. Centrifugal forces of the order of 15,000 to 20,000 times gravity readily displaced the pigment granules in the cortex of both the control and colchicine-treated eggs.

Fertilized eggs placed 10 minutes after fertilization in an 0.0002 molar concentration or stronger colchicine solution showed no mitotic figures or cleavage furrows developing. Weaker solutions of colchicine were observed to cause irregular cleavages, that is, the furrows grew in on the division plane from one side only. The mitotic apparatus in such cells was usually present but frequently showed evidence of being injured. Eggs that had undergone one division of the nucleus only in the colchicine solution when removed to normal sea water sometimes divided directly into 4 blastomeres or showed various types of abnormalities such as unequal division. Sections of such eggs frequently showed unequal separation of the chromosomes and multipolar spindles.

Fertilized eggs were allowed to stand 10 minutes after fertilization, at which time some were placed in a colchicine solution at two-minute intervals, extending over a period of 60 minutes. The eggs of all the samples were examined two to six hours later to determine the percentage that had undergone cleavage. The results of these experiments are given in Fig. 2.

It will be observed (Fig. 2) that no cleavages took place when the eggs had been allowed to develop not over 22 minutes (22°–24.4° C.) before being placed in the colchicine solution. However, if allowed to develop 28 to 36 minutes before being placed in the colchicine, at least 50 per cent of the eggs cleaved.

Cytological examination of the eggs placed in the colchicine solution 22 minutes after fertilization revealed them to be in the prophase stage of division. At 26 to 30 minutes after fertilization some were in late prophase, metaphase and anaphase stages of development. Attention should be called here to the work of Fry (1936) on the time schedule of mitotic changes in *Arbacia* eggs at Woods Hole. From his work, it will be observed that considerable variation occurs in speed

of development in *Arbacia* eggs even though they have all been taken from a single female. Furthermore, eggs taken from females at different months of the season were found to have different developmental tempos. Temperature is also a very important factor in the speed at which these eggs divide.

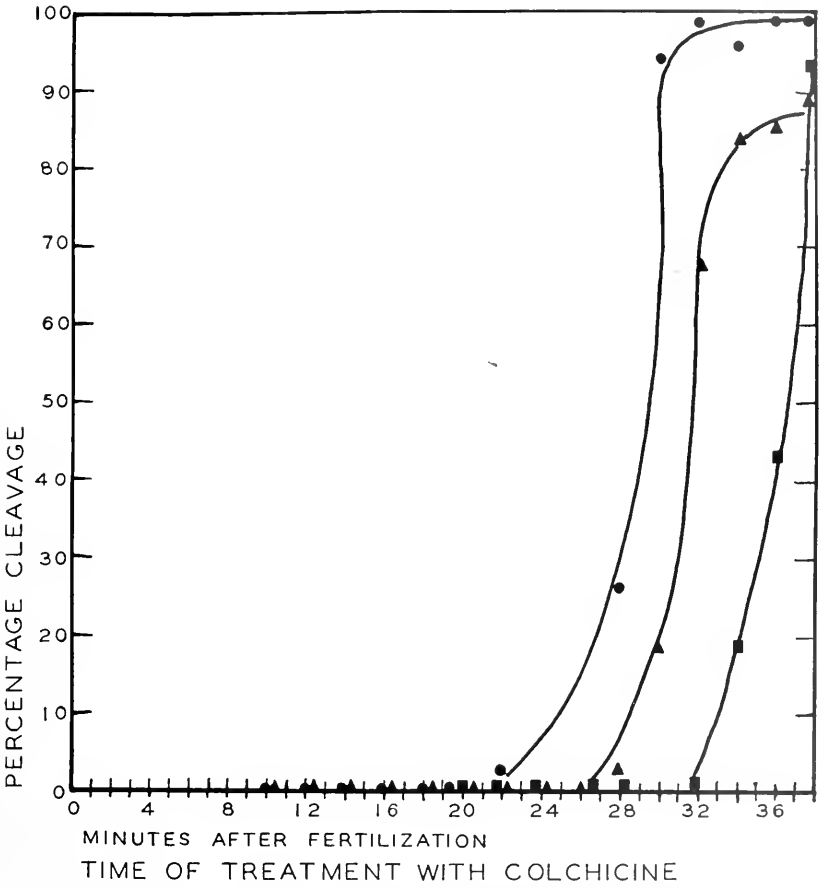

FIG. 2. A graph showing suppression of cleavage by colchicine. The results of three experiments are indicated by the curves. Note complete suppression until 22 minutes after fertilization. After this time the eggs rapidly became more resistant to the colchicine.

Dividing eggs that had been treated with colchicine were examined with a water immersion lens. The chief effect of the colchicine that could be detected by this method of study was upon the asters. As the colchicine penetrated the egg a gradual fading out of the astral rays occurred until eventually there appeared at each pole a clear

lake-like body. These, as we shall see later, represent the disintegration (liquefaction) of the asters.

In view of the fact that it is not possible to study accurately the chromosomes and mitotic apparatus in living sea urchin eggs, it was necessary to resort to fixed and stained preparations for this part of the study. Accordingly, we have recorded in Plate I some of the typical cytological changes that occurred following the colchicine treatment.

In Fig. 3 is illustrated a typical normal metaphase stage of division in *Arbacia*. It will be observed that the asters and chromosomes show clearly, while no indication of cleavage furrows is as yet to be observed. Figure 4 is a section through an egg taken from the same batch as that in Fig. 3 but placed in a colchicine solution at the same time that the one in Fig. 3 was fixed. After standing in the colchicine for 15 minutes the egg was fixed and stained in the same way as the egg in Fig. 3 had been. Here it will be observed that the asters have lost their rays, and have undergone an apparent liquefaction giving rise to two lake-like bodies in the cytoplasm. The chromosomes seem to be clumped, a condition that has been reported many times in various tissues following colchicine treatment. In this particular cell (Fig. 4) the cytoplasm appears to be more coarsely granular than in the normal egg but this is not a general characteristic of the colchicine effect, as may be noted by examination of Figs. 5 to 10. Figure 5 represents a section through an egg treated with colchicine while in the prophase stage of division. Here too the developing amphiaster has disintegrated, leaving a characteristic laked appearance in the cytoplasm. A much later stage in the mitotic cycle is illustrated in Fig. 6. In fact, cells advanced to this stage in the mitotic cycle sometimes cleave in spite of the colchicine treatment. However, here, as in the cases cited above, a disintegration of the amphiaster has taken place and the cleavage furrows have failed to form. On the other hand, Fig. 7 shows an egg from the same batch and treated in the same way as the one in Fig. 6 and apparently a normal separation of the chromosomes and cleavage has occurred. This might be explained by assuming that here a stage sufficiently late in the mitotic cycle had been reached where the asters had completed their chief function in the cleavage process before the colchicine was applied so that instead of blocking the cleavage mechanism, as occurs in the earlier stages of mitosis, it may have enhanced the process.

Various other types of cytological abnormalities following colchicine treatment will be seen in Figs. 8 to 10. In Fig. 8 two nuclei are seen which appear out of phase with each other so that the chromatin is not in the same physical state. The nucleus near the center of

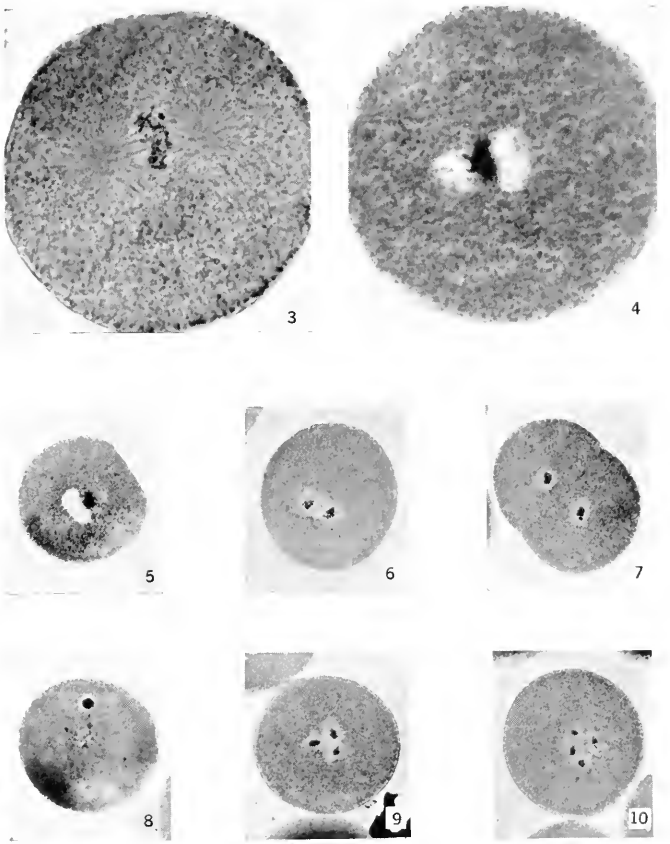

PLATE I

EXPLANATION OF FIGURES

All figures are photographs of *Arbacia* eggs fixed in Bouin's solution and stained in Heidenhain's hematoxylin. All except Fig. 3 have been treated with 0.0002 molar concentration of colchicine.

FIG. 3. Metaphase stage of control egg showing a typical mitotic figure.

FIG. 4. Egg in same stage as Fig. 3 when treated with colchicine. Note effects on the mitotic figure.

FIG. 5. Egg in prophase stage when treated with colchicine. A liquefaction of the spindle and asters have occurred as in Fig. 4.

FIG. 6. Later stage in the mitotic cycle showing destruction of asters and spindle after the chromosomes have separated. No cleavage furrows formed in this egg.

FIG. 7. Egg from same batch and treated in the same way as Fig. 6. Here the cleavage process occurred after the egg was placed in the colchicine solution.

FIGS. 8, 9 AND 10. Eggs showing various other types of abnormalities following colchicine treatment.

the cell seems to be in the early prophase; the one near the upper surface of the egg shows the chromatin clumped. Eggs that have been left in the colchicine solution for longer intervals have various abnormal groupings of their chromosomes, as indicated in Figs. 9 and 10, and also giant nuclei, an example of which is not shown in the plate.

Discussion

It is evident from the foregoing observations that the chief effect of colchicine upon the dividing eggs of *Arbacia* is to produce a lowering of the viscosity or at least a destruction of the gelation which normally occurs during division. This is accompaned by a rapid disintegration of the amphiaster and, when applied in the early stages of the mitotic cycle, an inhibition of the formation of the cleavage furrows. The chromosomes, however, may divide but usually they do not separate normally, thus producing either a restitution nucleus of a polyploid nature or several smaller nuclei in close proximity to each other. When the eggs are removed from the colchicine solution to sea water, many of the above-named changes are reversible.

Nebel and Ruttle (1938) have also mentioned the fact that colchicine in 10^{-4} molar concentration will block the cleavage process in *Arbacia punctulata*. In general our cytological observations upon the eggs following the colchicine treatment confirm theirs, but in their studies no efforts were made to correlate the cytological changes with the viscosity changes of the egg, or to determine at which particular stages in the mitotic cycle colchicine was effective in inhibiting the cleavage process.

Of the numerous agents (lipoid solvents, abnormal temperature, injury, drugs, hypertonic or hypotonic solutions, mechanical agitation, high hydrostatic pressure, etc.) that have been used to produce polyploidy in cells, none seems to be so effective and at the same time to produce so little harmful consequence to the cell as does colchicine. Because of this fact, it has been more successfully used by the geneticists to produce polyploidy in organisms than any other agent.

It is now definitely established from the works of Chambers (1917, 1924), Heilbrunn (1920, 1928), Fry and Parks (1934) and many others that in the course of cell division there is a marked physical-chemical change (viscosity) of the protoplasm associated with the different morphological changes (mitotic stages) in the cell. In fact, in the astral type of cleavage such as occurs in *Arbacia* the appearance of the asters and spindle seems to be an expression of a gelating action in the cytoplasm at these points which increases with the progressive development of the asters (Chambers, 1924; Heil-

brunn, 1928; Gray, 1931 and others). It is accordingly held by many that the asters are essential in the division mechanism of cells of this kind. This is substantiated by the fact that there are few, if any, authentic cases where cells that normally contain asters ever divide when these structures are absent or have been destroyed in the early stages of division.

The results recorded here substantiate this general view; in addition, an attempt has been made to determine at which stage in the mitotic cycle the amphiaster may be destroyed without blocking division of the eggs. In other words, is there a stage in the mitotic cycle before the appearance of the cleavage furrows in which the asters have completed their chief function in the division mechanism and after which time their rapid disintegration by colchicine will no longer completely block the formation of the cleavage furrows?

Our evidence seems to show that the critical point in mitosis, that is, the stage at which some of the eggs cleave and others were blocked when placed in the colchicine solution, occurred about 10 to 14 minutes before the appearance of the cleavage furrows of the controls when developing at temperatures between 22° and 24.4° C. Fixed, sectioned and stained eggs of this stage showed them to be in the late prophase, metaphase and a few in the early anaphase stages of division. It would seem then from this data that the abnormally rapid disintegration of the asters in *Arbacia* eggs once they have reached the late anaphase stage has little or no effect on the cleavage process. However, as pointed out above, destruction of the asters and the spindle in the prophase, metaphase or early anaphase blocks the cleavage process. It therefore appears that the asters and spindle are essential parts of the division mechanism of these eggs and that their main function in the process occurs in the early stages of the mitotic cycle.

It should be pointed out here that although the most generally accepted function of asters is that they play some kind of rôle in cell division, there are many animal cells which divide in the absence of demonstrable asters. However, whether or not a gel condition occurs at the poles of the spindles in such anastral mitosis has not been clearly demonstrated. Asters are usually found during mitosis in cells of large size although there are certain exceptions to this general rule. Fry (1934) has even suggested that they probably do not play any essential rôle in cellular activities.

The significant observations of Brown (1934) and of Marsland (1939) have demonstrated a marked variation in the viscosity of the cortex and interior of *Arbacia* eggs prior to and during cleavage. By means of high hydrostatic pressure they have been able to produce a

liquefaction of the cortex of dividing eggs so that no furrow forms and previously formed but incomplete furrows undergo recession. Preliminary observations seem to indicate that the amphiasters and spindle of such eggs were relatively unaffected by the pressure treatment.

To what extent the colchicine solution affected the viscosity of the surface of the egg was not so easily determined as was its effect upon the interior. However, our observations show that a slightly greater distortion and displacement of the pigment granules seemed to occur in the colchicine-treated eggs than in the controls. This would indicate at least a slight lowering of the viscosity of the surface of the egg, although it is not sufficiently great to inhibit the completion of the cleavage process once the furrows have started to form, differing here from effects of hydrostatic pressure. Furthermore, colchicine does not inhibit locomotion in *Amoeba* (unpublished observations) or cyclosis in the cells of *Tradescantia* (Nebel and Ruttle, 1938), both processes of which are thought by some to depend upon the sol $\rightleftarrows$ gel reactions at the surface.

Our work and that of others (Heilbrunn, 1915, 1920, 1928; Chambers, 1924, 1938; Brown, 1934; Gray, 1931; and Marsland, 1939) suggests that the division process in *Arbacia* eggs is associated with two separate but usually correlated processes (sol $\rightleftarrows$ gel reactions) occurring on the interior and at the surface of the eggs, the former being associated with the formation of the mitotic figure and the latter with the formation of the cleavage furrows. Experimental work has shown that the time of appearance of these reactions in the division process is of fundamental importance. A sol reversal of the mitotic figure by colchicine or mechanical agitation during the prophase or metaphase stage inhibits the formation of the cleavage furrows, while a sol reversal of the cortex at this time by mechanical agitation has no effect (Chambers, 1938). However, a few minutes before and during the formation of the cleavage furrows a reversal of the mitotic figure to the sol state by colchicine or by mechanical agitation has little or no effect on cleavage while a reversal of the cortex to a sol state at this time by mechanical agitation or hydrostatic pressure blocks the process (Chambers, 1938; Marsland, 1939).

The work with colchicine on dividing eggs seems to support the view that has been expressed by others, namely, that the spindle is essential for the proper separation of the chromosomes and that conditions which bring about a solation of the cytoplasm cause a destruction of the spindle while the chromosomes may be relatively unaffected.

Conclusions

1. Colchicine in concentrations of 0.0002 molar or stronger will inhibit cleavage in *Arbacia* eggs developing at 22° to 24.4° C. if applied before approximately 22 minutes after fertilization. Cytological observations have shown that eggs placed in the colchicine solution while in the prophase, metaphase or early anaphase stages are prevented from completing their cytoplasmic division.

2. The chief action of the colchicine upon the dividing eggs is to lower the viscosity or to inhibit and destroy the increased gelation that normally occurs within the eggs during division. Evidence for such action was determined by comparing under similar conditions the stratification of materials on the interior of control and colchicine-treated eggs. Colchicine also seems to lower slightly the consistency of the periphery of the eggs but not enough to cause a disintegration of the cleavage furrows once they have started to form.

Unpublished observations also show that colchicine does not inhibit locomotion in *Amoeba* which is thought to depend upon a sol $\rightleftarrows$ gel reaction at the surface, as is also the formation of cleavage furrows.

3. Colchicine produces a reversal of the asters and spindle to the sol state accompanied by a disappearance of the astral rays and spindle fibers. The chromosomes divide but do not separate normally due presumably to the loss of the directing force of the spindle.

4. The evidence obtained seems to substantiate the view that in the astral type of cleavage, such as occurs in *Arbacia*, the asters play an important part in the mechanism of cell division. Their destruction at any time before late anaphase seems to inhibit cleavage. However, in later stages of division an acceleration of the return of the asters to a sol state by colchicine does not inhibit the completion of the cleavage process. This indicates that the main rôle of the asters and spindle in the cleavage process occurs before the late stages in the mitotic cycle.

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DECOMPOSITION OF CHITIN BY MARINE BACTERIA

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Chitin is an important constituent of the exoskeletons of many marine animals. For example, the shell of the horseshoe crab, *Limulus polyphemus*, consists on an average of 25 per cent chitin. Due to the death of marine inhabitants, and to the production of chitinous casts during the developmental stages of certain species, large quantities of chitin are released annually. If this material were not decomposed, much carbon and nitrogen would be withheld from the cycles of these elements in the sea. Analyses of marine sediments indicate, however, that relatively little chitin accumulates there; it must, therefore, be broken down into simpler chemical compounds, or mineralized.

Decomposition of chitin by mixed or pure cultures of marine bacteria has been demonstrated by several investigators (2, 4, 12, 14, 16, 17), whose observations indicate that chitin-decomposing bacteria have a worldwide distribution in the sea. The present investigation was undertaken to study the distribution of these bacteria in a relatively restricted region of the sea, and to follow the breakdown of chitinous materials in natural media from this same locality.

Chitin has an elementary composition of $C_{32}H_{54}O_{21}N_4$ (6). It is a tough leathery substance, insoluble in water, in concentrated alkalis and in the usual organic solvents. By hydrolysis with concentrated acid it yields glucosamine and acetic acid in proportions which indicate that there is an acetyl group for each glucosamine residue. So far no essential differences have been found in the chitin from animal or vegetable sources (5, 7).

Isolation of Chitin-Decomposing Bacteria

Purified chitin, used in making culture media for the detection and isolation of chitin-decomposing bacteria, was prepared from horseshoe crab shells by the methods of Benton (1) and ZoBell and Rittenberg (17). The shells were treated successively, for prolonged periods, with 1 per cent hydrochloric acid, 2 per cent potassium hydroxide and 95 per cent ethyl alcohol. The snowy-white material which remained reacted positively to simple qualitative tests for chitin (3).

[1] Contribution No. 231.

Liquid culture media were prepared by partly covering 1 × 5 cm. strips of purified chitin, in test tubes, with sea water, or with sea water containing 0.1 per cent concentrations of additional nutrients such as dextrose, peptone, or ammonium chloride. In some cases a small amount (0.005 per cent) of phosphate was added to the sea water to eliminate any effect due to lack of available phosphorus. The medium best suited for the detection of chitin-decomposing bacteria consisted of a strip of chitin partly covered with a solution used by Waksman (14), and having the following composition:

Glucose	1 gram
Peptone	1 gram
K_2HPO_4	0.05 gram
Sea water (filtered)	1,000 ml.

Solid media were prepared from the liquid culture media either by the addition of 15 grams of agar to each liter of solution or by diffusion of the nutrient solutions through silica gels. Where solid media were employed strips of sterile purified chitin were placed on the surface of the hardened gel. The silica gel plates were prepared under as nearly aseptic conditions as possible; all other media were sterilized by autoclaving for 20 minutes at 120° C.

One additional solid medium was found useful for the rapid detection of chitin-decomposing bacteria. It was prepared by uniformly dispersing chitin in agar according to the following procedure. Fifteen grams of purified chitin were added to 150 ml. of 1 : 1 sulfuric acid and kept overnight in the refrigerator. The chitin was dissolved by this treatment but analyses showed that it was not altered chemically, i.e. hydrolyzed. The following day the chitin was precipitated by the addition of approximately 140 ml. of 20 per cent potassium hydroxide. The precipitate was placed on a Büchner funnel and washed repeatedly with distilled water until the filtrate was neutral to litmus. Thirty-five grams of the chitin, as removed from the filter, were then added to agar which contained 0.1 per cent glucose, 0.1 per cent peptone, and 0.005 per cent dipotassium hydrogen phosphate. After autoclaving, the agar was thoroughly shaken to redistribute the chitin, and Petri plates were prepared. In this condition the medium was of a uniform milky-white opacity. When chitin-decomposing bacteria grew on the surface of this agar they dissolved the chitin, thereby producing a clear halo around each colony. By noting the length of time necessary for the appearance of halos the rates of bacterial activity could be estimated. Bacteria which decomposed chitin rapidly often produced a halo after two days' growth whereas others required eight to ten days for a positive test. Although bacteria which were

unable to decompose chitin often grew well on the medium, they did not alter it in appearance. Results obtained with this agar confirmed, without exception, the results obtained with other solid and liquid media.

In order to test a given sample of marine material for chitin-decomposing bacteria, several different media were inoculated. The inoculated media were incubated at room temperature and examined periodically for evidence of chitin breakdown. Growth was slow in the medium consisting of only chitin in sea water, whereas the same bacteria decomposed the chitin more rapidly when additional nutrients were present.

Visible dissolution of the chitin, either in liquid medium or on a solid agar surface, was indisputable evidence that the chitin was being attacked. To detect incipient decomposition a hand lens was helpful. The liberation of ammonia from media lacking peptone or nitrate, reducing substances from media lacking glucose, or a pronounced change in acidity, were further reliable evidence of chitin decomposition. The mere growth of bacteria in a medium consisting of only chitin in sea water is an unreliable criterion of decomposition, since bacteria may persist in this medium for a long period without altering the chitin.

In preliminary studies, cultures of bacteria showing no evidence of chitin-decomposition were kept one year before being discarded as negative. It became evident, however, that several weeks were adequate for detecting the chitin-decomposing capacity of most species. To obtain pure cultures of bacteria a second tube of enriched medium was inoculated with a loopful of material from an original positive culture. Growth from this tube was then streaked on nutrient agar plates and the different types of colonies which developed were retested for their ability to attack chitin. In later experiments pure cultures were obtained more quickly by streaking growth from enriched cultures on agar medium containing precipitated chitin. This medium was highly satisfactory for separating the chitin-decomposing bacteria from others which had persisted but which were not attacking the chitin.

Occurrence of Chitin-Decomposing Bacteria in the Sea

The number of chitin-decomposing bacteria in sand, mud, and water was determined by dilution culture methods. To increase the probability of detecting chitin-decomposing bacteria, several kinds of culture media were inoculated with each dilution of the sample. In making quantitative determinations only visible breakdown of the chitin was considered a positive test.

An 11 cm. core of sand, taken from a littoral zone in the vicinity of Woods Hole harbor on July 31, 1939, was found to be a relatively rich source of chitin-decomposing bacteria. At the top of the core there were at least 60,000 of these bacteria per gram of sand, this number decreasing to 6,000 per gram at a depth of 5 cm., and to 600 per gram at 11 cm.

A sample of mud was collected on July 7, 1939, at latitude 40° 38′ N. × 71° 39′ W. and at a depth of 878 meters. Chitin-decomposing bacteria were relatively abundant in the upper layer of the core (125 cells per gram), but dropped to less than 5 cells per gram at depths of 5 and 11 cm. Both in sand and in mud the number of chitin-decomposing bacteria decreased sharply with core depth.

On August 2, 1939, a sample of water was collected at 5 fathoms NE. of Lambert's Cove, Martha's Vineyard, one mile offshore, in a locality where the water is naturally well stirred. Dilution culture methods indicated the presence of 150 bacteria per ml. of water.

Chitin-decomposing bacteria were isolated from other samples of sand, mud and water, in addition to those described above. There was no correlation noted between abundance of the bacteria in the sediments and depth of the overlying water. In addition to bacteria isolated from media collected in the vicinity of Woods Hole a few cultures of chitin-decomposing bacteria were isolated from samples of water brought from tropical regions by the research ship "Atlantis."

Dead and decomposing horseshoe crabs offered an excellent source of chitin-decomposing bacteria. A single attempt to isolate these bacteria from a jar of dead copepods was unsuccessful.

That animals may possess intestinal fauna and flora capable of breaking down certain foods ingested by the host is well established. It has been demonstrated, for example, that wood-boring termites harbor in their intestinal tract microörganisms which perform for their host the invaluable function of digesting cellulose (15). Since a similar condition in relation to chitin might exist in marine organisms, the intestinal contents of several common marine animals were examined for chitin-decomposing bacteria. Using methods already described for their detection, chitin-decomposing bacteria were found in the intestinal contents of *Venus mercenaria mercenaria*, *Ostrea virginica*, *Loligo pealeii pealeii*, *Ovalipes ocellatus ocellatus*, *Mustelus mustelus*, *Raia erinacea* and *Spheroides maculatus*. In this investigation, all animals whose intestinal contents were examined for chitin-decomposing bacteria were found to possess them. The presence of these bacteria in the intestines of marine animals has previously been demonstrated by Benton (1), who isolated them from the speckled

trout and by ZoBell and Rittenberg (17), who found from one hundred to more than a thousand chitin digesters in each milliliter of the stomach contents of the squid.

There are few marine animals which do not ingest chitin if they come in contact with it. According to available information (8, 9, 10), however, the oyster does not take in chitin at any time. Accordingly, it is interesting to note that chitin-decomposing bacteria were found in the alimentary canal of this animal, also.

Decomposition of Limulus Shell in Natural Media

Having demonstrated the presence of chitin-decomposing bacteria in marine habitats, it was decided to follow the breakdown of a naturally-occurring chitinous material in several marine media. Accordingly, *Limulus* shells were scrubbed free of flesh, cut into strips approximately 3 × 8 cm., weighed (about 3 grams after drying overnight at 100–105° C.) and placed in marine sand, mud and sea water. Sand from the littoral region was scooped up, to a depth of 15 cm., and thoroughly mixed. Weighed strips of crab shell were placed in glass jars and covered with 200 grams of the sand. Likewise, strips of *Limulus* shell were covered with bottom mud collected in the Woods Hole harbor. Sea water, collected a few feet from the shore, was placed in bottles in 200 ml. volumes. Weighed strips of crab shell extended about 1 cm. above the water level. All cultures were kept in a dark cabinet at room temperature. Occasionally distilled water was added to the sand and mud cultures to prevent them from becoming dry.

These experiments were continued for 57 weeks. At stated intervals one culture from each of the three series (sand, mud, water) was examined. The crab shell was removed, weighed and checked for visible evidence of decomposition. Any changes in color and texture of the natural media were noted. The sand and the mud were analyzed for total organic carbon and nitrogen. The carbon was determined by Tiurin's modification (13) of Schollenberger's method (11). The nitrogen determinations were made by the usual Kjeldahl procedure. The results of the analyses are based upon the total dry sand or mud (dried overnight at 100–105° C.) not freed from the sea salt.

In sand, decomposition of the *Limulus* shell began at once. At the end of one year, when the experiment was discontinued, the shell had lost about three-fourths of its original weight (Fig. 1) and had been reduced to a small fraction of its former size. At the beginning of this experiment the sand contained 0.8 per cent carbon, and 0.05 per cent nitrogen, indicating that its organic content was relatively low. Al-

though a large amount of organic material was decomposed during the course of a year, there was no measurable increase in carbon and nitrogen in the sand. Presumably the compounds liberated from the crab shell were used in the metabolism of the bacteria.

When *Limulus* shell was placed in bottom mud, decomposition of the shell, as measured by decrease in weight, was detectable only after a period of about six weeks. Thereafter the shell decomposed fairly rapidly until at the end of one year it had lost a third of its weight (Fig. 1). The original mud was high in organic content. When brought into the laboratory it contained 2.4 per cent carbon and 0.17 per cent nitrogen. During the course of decomposition of the crab

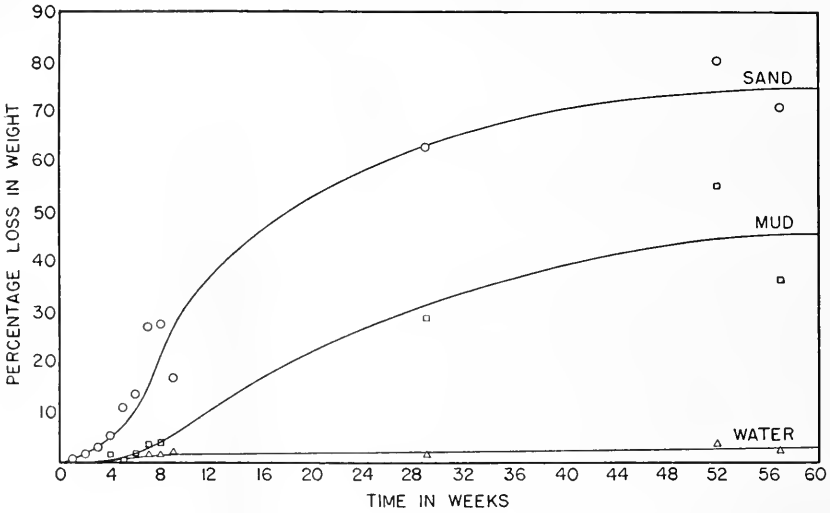

FIG. 1. Decomposition of horseshoe crab shell in natural marine media.

shell over a period of one year, the amount of carbon in the mud remained unchanged, while the nitrogen increased from 0.17 to 0.22 per cent.

In sea water there was only a very slight decrease in weight of the crab shell, even after a period of more than a year (Fig. 1). At the end of this time the shell was practically unchanged in appearance. This need not mean that chitin-decomposing bacteria were absent from the sea water, but only that under the experimental conditions there was no marked breakdown of the chitin in this medium, whereas in sand and in mud, under comparable conditions, the decomposition was pronounced. As far as laboratory experiments can show, chitin appears to be decomposed more slowly by marine bacteria than are most other structural organic materials.

During decomposition the shell underwent striking physical changes. Originally tough and leathery, it became soft and fragile as large areas were removed. Incipient decomposition was detected by using a binocular microscope, when the shell was observed to be "eaten" away in spots. In advanced stages of decomposition the shell was greatly reduced in size as well as in weight. In preparing purified chitin from *Limulus*, it was noted that continuous sheets of the purified material were obtained from all parts of the shell. It follows, therefore, that as the latter decomposed in these natural marine media, the chitin was digested along with the other compounds which make up the horseshoe crab shell.

The detailed characteristics of these bacteria are now being investigated. Preliminary tests indicate that approximately fifteen types of bacteria have been isolated. They are Gram negative, asporogenous, motile rods. Among the readily demonstrable products resulting from the decomposition of purified chitin by pure cultures of the bacteria are ammonia, reducing substances, and organic acids.

Summary

Chitin-decomposing bacteria were isolated from the following sources: marine sand, mud, water, decomposing horseshoe crabs, and the intestinal contents of *Venus mercenaria mercenaria*, *Ostrea virginica*, *Loligo pealeii pealeii*, *Ovalipes ocellatus ocellatus*, *Mustelus mustelus*, *Raia erinacea* and *Spheroides maculatus*.

Chitin-decomposing bacteria were found to be most abundant in marine sand, less abundant in mud, and present in small numbers in sea water.

Under experimental conditions, the decomposition of *Limulus* shell was relatively rapid in littoral sand, moderate in bottom mud, and negligible in sea water.

The writer is indebted to Dr. Selman A. Waksman for his interest and helpful suggestions.

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THE EFFECTS OF IMPLANTING ADULT HYPOPHYSES INTO SEXUALLY IMMATURE FUNDULUS

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The factors which control the sex cycle of vertebrates are becoming increasingly clear as a result of numerous investigations. There is gratifying agreement, among all workers on animals above fishes in the evolutionary scale, that the pituitary gland plays a dominant rôle in the direct control of periodic changes that occur in the gonads and associated sexual organs. Control of the sexual cycle in fishes, however, is little understood. Efforts to determine the rôle which the pituitary gland may play in controlling this cycle have yielded conflicting results, particularly with reference to the effect of injecting mammalian pituitary extracts or pituitary-like preparations. Thus Calvet (1932) and Damas (1933) succeeded in stimulating the ovaries of lampreys with prolan and extracts of the mammalian pituitary gland; Boucher, Boucher and Fontaine (1934) obtained an enlargement of the gonads of both sexes in eels which were injected with pregnancy urine and saw evidence of maturation of sperm in the testis; and Morosowa (1936) found that when either pregnancy urine or prolan was injected into perch, laying of eggs and ejaculation of sperm could be produced at any time during the winter. On the contrary Koch and Scheuring (1936) using prolan, Matthews (1939) with injections of sheep pituitary extract and Johnson and Riddle (1939) employing three different mammalian extracts, all of which were shown to be active in stimulating the gonads of birds and mammals, obtained no such effect when these materials were injected into fishes. Fairly uniform results have been obtained, however, by injecting fish pituitary preparations into other fishes. Houssay (1930, 1931), Cardoso (1934), Pereira and Cardoso (1934), von Ihering (1935), and Azevedo, Dias and Vieira (1938) have all observed that the injection of saline suspensions or extracts of fish pituitary glands into other fishes resulted in an enlargement of the gonads and frequently in expulsion of eggs and sperm as well. In the work of von Ihering and of Azevedo, Dias and Vieira mating activities were also elicited in this way. More recently

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Hasler, Meyer, and Field (1939) found that when pituitary extracts of carp were injected into trout, mature eggs and sperm were produced six to seven weeks before the normal breeding season. Further evidence that the pituitary gland may be of importance in controlling changes in the gonads of fishes has been obtained by removing the gland. Vivien (1938, 1939) found that after removal of the hypophysis from *Gobius* marked regressive changes occurred in both testis and ovary; and Matthews (1939) showed that when male *Fundulus* were hypophysectomized and kept until the next breeding season the testes did not enlarge and become active as did those of control animals, and that no sperm and very little spermatogenic activity were present in the operated animals.

Work so far carried out on fishes, then, indicates that the fish pituitary gland contains some material capable of modifying the normal sex cycle, and suggests that in these forms, as in other vertebrates, the hypophysis is of importance in controlling changes in the gonads and associated organs. If this be true, then the injection of teleost pituitary extracts or the implantation of fresh preparations might be expected to produce an effect on the gonads of sexually immature animals, stimulating them to develop earlier than they normally would. The following is a report of experiments carried out on sexually immature individuals of *Fundulus* to determine whether or not precocious development of the gonads could be produced in this way.

An adequate supply of small *Fundulus* was collected in the small salt water streams on Penzance Point, at Woods Hole, by means of a small trap. A number from each group collected were killed immediately and their gonads examined. Of 136 animals examined in this way, ranging in length from 22 to 40 mm. from the tip of the snout to the base of the caudal fin, only 4 of the 65 males (lengths 32, 34, 39 and 40 mm.) showed whitish, active testes with sperm in fresh smear preparations, characteristics of the adult male during the breeding season. The remaining 61 males had testes that were definitely inactive. All 71 of the young females showed only small ovaries with no large follicles. Obviously the vast majority of these small animals were sexually immature.

Attempts were made to stimulate the gonads of these immature animals in two ways. In the first series of experiments the pituitary gland of a sexually mature *Fundulus* was implanted into the eye of an immature animal. From the success achieved with this method in mammals it was hoped that the transplanted gland would maintain itself and provide a continuous supply of any hormones produced by it to the host. From July 25th to August 5th 95 such implants were

made. The animals were kept in tanks provided with running sea water and a number of untreated young animals were placed in adjacent tanks for controls. Both groups were fed two or three times a week with fresh clam meat. Three experimental animals with controls of the same lengths were killed, two four weeks after implantation of the adult hypophysis and one five weeks following this operation. Of two experimental females only one showed a marked enlargement of the ovary over those of the controls, but microscopic sections showed that in both animals the ovarian follicles and their contained eggs were much larger than those of the control ovaries and that they were provided with larger amounts of vesicular yolk. The testis of the implanted male was also large, was whitish in color, and showed many sperm both in fresh smears and in microscopic sections; while that of the control male, like that found in the majority of untreated males of this size, was small, practically colorless and showed no sperm. The implanted pituitaries were recovered from the male and from one of the females. Sections of them showed fairly normal cells. In only one of them (that of the male) could blood vessels be traced from the tissues of the eye to the implanted gland.

The remaining animals were lost in an attempt to transport them from Woods Hole to Williamstown at the end of the summer.

The following summer a second series of the same sort was run. During the last week in July implants of adult hypophyses were made into the right eyes of 185 young animals, with 112 others serving as controls. For some undetermined reason there was a high mortality in this series. Only 12 males and 12 females of the experimental animals and 38 males and 37 females of the controls were still alive during the first week in September when they were killed and examined. In the females no effect of the implants on the ovary was observed in any case. Of the experimental males only 1 animal, 32 mm. in length, showed an active testis. The implanted pituitary glands were recovered from a number of these animals. Differences between them and the glands recovered from the previous series, where a certain amount of stimulation of the gonads had apparently occurred, were not striking, although in this latter series the cells were somewhat more shrunken and fewer granules were present in the cytoplasm.

Thus of the 27 young animals examined after implanting an adult pituitary gland into the eye, increased activity of the gonads over those of controls was apparent in only four. Since untreated animals of this size occasionally show similar development of the gonads (as in the four males with active testes mentioned earlier), it was certainly not

clear that the observed increase in activity of the gonads of these 4 experimental animals had been produced as a result of the implantation of the adult gland. In fact, the failure of the other 23 cases to show any effects suggested rather that the implanted glands lacked effective sex-stimulating hormones. It was possible, however, that the transplanted glands did not establish themselves successfully in the eye and that as a result such hormones as they might normally produce were delivered to the circulation for too short a time to be effective. In order to determine which of these possibilities was correct a second method of implanting the adult gland was employed. Preliminary experiments showed that when the pituitary gland of an adult was placed in the peritoneal cavity of a sexually immature animal through a small slit in the body wall the gland could be recovered in an apparently normal condition four or five days later. Twenty-seven young animals were treated in this way. Since the sex of the young animals could not be distinguished, no attempt was made to select the sex of the donors. As in the previous series the experimental animals and controls of appropriate sizes were kept in running sea water in adjacent tanks, both being fed twice a week with fresh clam meat. At three-day intervals each of the experimental animals received a fresh pituitary gland from an adult animal. Such serial implants should provide the immature animal with a fairly constant supply of whatever hormones might be liberated by the adult gland. These animals withstood the handling necessitated by the operations very well. The series was begun on August 8th and ran until September 3rd, during which time only three of the experimental animals died. Records were thus obtained on 24 animals, 10 females and 14 males. In every case the gonads of the experimental animals were much larger and more mature than those of the controls. The females showed not only an increase in size of the whole ovary (Fig. 1) but a marked increase in size and maturity of the eggs (Figs. 2 and 3). The effects on the males were even more marked. The testes of all experimental animals were white and very active, with a high degree of spermatogenic activity as shown in microscopic sections (Figs. 4, 5 and 6). Moreover, in these experimental males the dorsal fin had a prominent black pigment spot which several workers (Newman, 1908; Chidester, 1917; Parker and Brower, 1935) have shown is associated with sexual activity in adult males (Fig. 7).

From these experiments it is clear that when pituitary glands of sexually mature *Fundulus* are implanted into an immature animal in such a way as to provide a continuous supply of fresh gland material, pronounced stimulation of the gonads of both sexes is produced. The

evidence obtained from these experiments coupled with that previously obtained by removing the gland in adult males indicates that the pituitary gland of *Fundulus*, like that of vertebrates above fishes in the evolutionary scale, contains a sex-stimulating substance, presumably a hormone, which is of importance in controlling the development and subsequent seasonal changes in the gonad of this teleost.

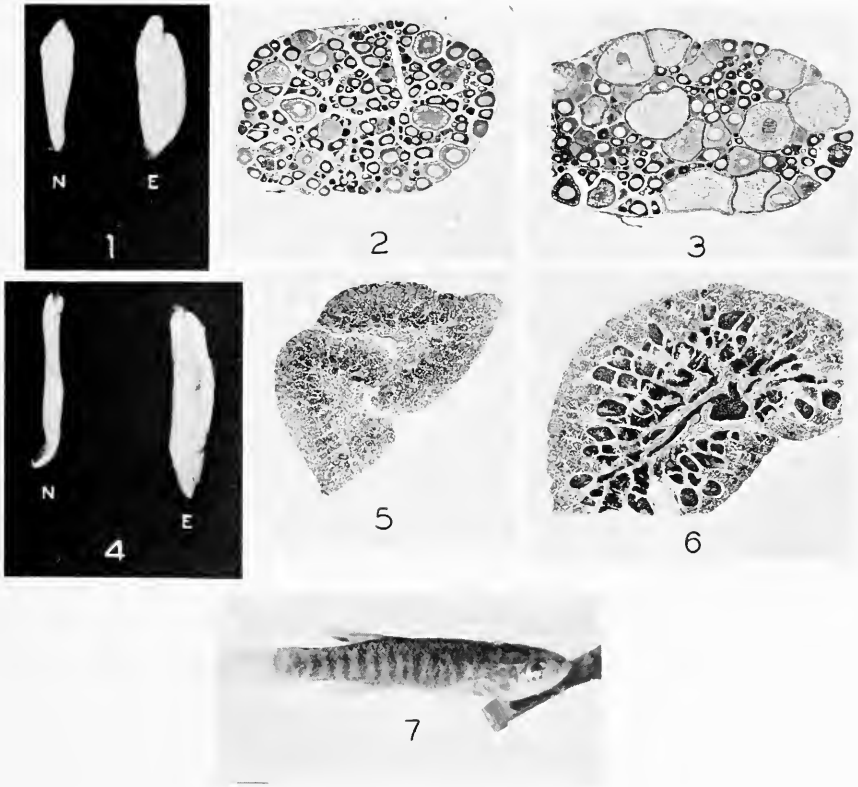

FIG. 1. Ovaries of young *Fundulus*, ×2.5. *N*, ovary of normal animal, 32 mm. long; *E*, ovary of female of same length which had received six adult pituitary glands over a period of 26 days.

FIG. 2. Cross-section of normal ovary shown in Fig. 1, ×25.

FIG. 3. Cross-section of experimental ovary shown in Fig. 1, ×25.

FIG. 4. Testes of young *Fundulus*, ×2.5. *N*, testis of normal animal, 37 mm. long; *E*, testis of male of same length which had received six adult pituitary glands over a period of 26 days.

FIG. 5. Cross-section of normal testis shown in Fig. 4, ×45.

FIG. 6. Cross-section of experimental testis shown in Fig. 4, ×45.

FIG. 7. Experimental male, 23 mm. long, in which the testis had been activated by implanting adult pituitary glands. Note pigment spot on dorsal fin.

That this is true of other teleosts as well is indicated by the work on other forms already reviewed. The evidence is quite clear that the teleost pituitary gland, when suitably injected or implanted, will stimulate activity in the gonads of other fishes. While the evidence obtained from injecting mammalian pituitary preparations is not as clear because of conflicting results, certainly most of the recent work shows that such mammalian preparations are either relatively or completely ineffective in producing gonadal stimulation in fishes. These facts suggest that a certain degree of zoölogical specificity of the pituitary hormones may exist, a point of view which has recently been discussed at some length by Creaser and Gorbman (1939). In this connection it is of interest to note that mammalian sexual hormones of both sexes, unlike mammalian pituitary preparations, have produced quite consistent results. Thus Fleischman and Kann (1932) showed that the injection of progynon into adult *Rhodeus* during the inactive period of the sexual cycle led to development of the oviduct comparable to that found during the breeding season; Berkowitz (1937, 1938) also showed that the feeding of progynon tablets to *Lebistes*, beginning at birth, caused a suppression of male secondary sexual characters and a marked suppression of spermatogenic activity, without, however, producing any effect on the secondary sexual characters of the female; and Eversole (1939) showed that the injection of testosterone propionate into *Lebistes* suppressed both body growth and ovogenesis in the female and induced the development of some male secondary sexual characters; while Regnier (1939) caused male secondary sexual characters to appear in females of both *Lebistes* and *Xiphophorus* and produced degenerative changes in the ovary by injecting testosterone propionate or merely by adding it to the water of the aquarium in which the animals were kept. Whatever factors are responsible for the lack of response met with when mammalian pituitary preparations were applied to fishes apparently do not apply to mammalian estrogenic and androgenic substances.

Summary

Pituitary glands of adult *Fundulus* were implanted into the eyes of sexually immature animals. Of twenty-seven cases examined several weeks after operation the gonads of only four showed greater activity than did those of control animals.

In another series of experiments adult pituitary glands were implanted at three-day intervals into the peritoneal cavity of immature *Fundulus*. At the end of four weeks all twenty-four experimental animals of both sexes showed a high degree of gonadal stimulation.

This was particularly notable in the males where large numbers of mature spermatozoa and pigmentation characteristic of the adult during the breeding season were produced. The success of this method of implantation, which continuously supplied the young individual with fresh gland material, indicates that the failure in most cases of the method employing intra-ocular implants was due to a failure of the implanted gland to establish itself successfully so that it provided an inadequate supply of gonad-stimulating substances to the host.

The precocious development of the gonads produced by implanting the adult pituitary gland strongly suggests that the normal development of the mature condition of the gonad in *Fundulus* is controlled by the pituitary gland.

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THE TRANSPORT OF OXYGEN BY THE BLOOD OF FRESHWATER FISH

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Introduction

The stresses imposed by low oxygen pressures upon the ability of fish to oxygenate their blood and by low temperatures upon their ability to utilize oxygen in the blood were first appreciated by Krogh and Leitch (1919). In their study of four species of freshwater fish and two marine species they noted the apparent relationship between the pressure of oxygen required to oxygenate the blood and the pressure of oxygen in the external environment in which the species live. They stated that the difference in the properties of the blood (between fish living in poorly oxygenated water and well oxygenated water) was of such a magnitude as to suggest species differences. They also noted that carbon dioxide had a greater effect on the blood of fish than on mammalian blood in reducing the affinity of the hemoglobin for oxygen. This effect, known as the "Bohr effect" is thought to be an advantage in the transport of oxygen in that it should raise the pressure at which oxygen is discharged from the blood and thus should facilitate diffusion of oxygen to the tissues. The latter property, namely the enlarged Bohr effect, was considered by Krogh and Leitch as an adaptation to low environmental temperature, while the possession of a higher affinity to oxygen was regarded as an adaptation to low environmental oxygen tension. They suggested that these adaptations were made possible by special conditions within the erythrocyte affecting the hemoglobin.

Root (1931), in his careful examination of the blood of several marine teleosts, found that carbon dioxide reduced the maximal amount of oxygen combined, even in the presence of as high a pressure of oxygen as 150 mm. Hg. He found too that the blood of each species was very sensitive to carbon dioxide compared with mammalian blood, but his data do not indicate that the affinity of the hemoglobin varies significantly from species to species. Willmer (1934), in a study of the respiration of tropical freshwater fish, noted amongst other features that the blood varied in sensitivity toward carbon dioxide. He observed a relation between the environment of the fish and the sensi-

tivity of the blood towards carbon dioxide, the allegedly active river fish having the most sensitive blood and the fish of the swamp possessing the least sensitive blood. Powers *et al.* (1932, 1938) have presented some data on oxygen dissociation of freshwater fish blood and on the relation of environmental gases to blood gases. Black and Irving (1938) showed that the ability of carbon dioxide to alter the affinity of blood of certain fish depends largely upon the intact corpuscle.

The present paper deals with certain characteristics of the blood of four species of freshwater fish, which determine the capacity of the blood of the fish for the transport of oxygen. This has been accomplished by the construction of oxygen dissociation curves for whole blood at various pressures of carbon dioxide and at one temperature (15° C.). Comparison of the properties of the blood can only be made at the same temperature, and the temperature chosen was one at which all the species could survive with apparent ease. This allows the correlation of the properties of the blood with the environmental characteristics of the fish, but restricts deductions as to the natural functioning of the blood of each species in its characteristic environment whatever that may be.

The results obtained show specific differences in the affinity of the hemoglobin and, moreover, specific differences in the sensitivity of the whole blood to carbon dioxide, an effect which has been shown (Black and Irving, 1938) to depend in a large degree upon the intact erythrocyte. In addition, data are presented which show that blood of high oxygen affinity has a low sensitivity to carbon dioxide, and blood of low oxygen affinity possesses a high sensitivity to carbon dioxide.

Materials and Methods

In this study four species of freshwater fish were used. These represented two superorders, Ganoidei and Teleosti of the subclass Actinopteri. The ganoid fish investigated was the bowfin, *Amia calva* Linnaeus. The teleost fish employed were the common sucker, *Catostomus commersonnii* (Lacépède), the carp, *Cyprinus carpio* Linnaeus and the catfish, *Ameiurus nebulosus* (Le Sueur).

The bowfin were obtained from the Great Lakes while the common suckers and carp were caught in Ontario lakes (Scugog and Erie). The catfish were provided by the Torresdale fish hatchery (Delaware river).

Blood was drawn from the heart by means of a Luer syringe. In the case of the three larger species, blood from a single fish was used for a number of curves. Dry heparin was added to prevent clotting. In no instance was any substance employed to prevent the normal respiration of the cells.

Immediately after the blood was drawn it was transferred to a tonometer of the type described by Irving and Black (1937). The gas mixtures were made up manometrically and the blood-gas mixture equilibrated in a water bath for not less than 20 minutes at 15° C. Emphasis should be placed on this fact, that only the drawn blood was equilibrated at 15° C. and not the fish. The common suckers and carp were taken from water, the temperature of which varied from 5° to 15° C.

The size of the sample of blood used for each analysis was 0.5 ml. Oxygen and CO_2 were determined by means of the "combined method" of Van Slyke and Neill. The reaction mixture was shaken in the manometric Van Slyke apparatus for at least 6 minutes, for oxygen is

TABLE I

The oxygen capacity and red cell volume of the blood of four freshwater fish

Species	Number of fish	Oxygen capacity vol. % HbO_2	Red cell volume %	Oxygen capacity 100 ml. cells vol. % HbO_2
Catfish, *Ameiurus nebulosus*	15			
Mean		13.3	32.2	41
Range		8.4–17.4	20.5–39.7	34–46
Carp, *Cyprinus carpio*	16			
Mean		12.5	33.1	37
Range		6.4–22.9	16.4–61.0	34–44
Bowfin, *Amia calva*	5			
Mean		11.8	27.1	44
Range		8.5–13.9	25.6–30.7	42–45
Common sucker, *Catostomus commersonnii*	39			
Mean		10.6	32.5	33
Range		5.9–14.6	20.4–48.5	21–43

often only slowly extracted from fish blood. The agreement between two consecutive determinations of oxygen capacity was within 0.3 volumes per cent. The gas phase was analyzed in a Haldane apparatus.

The factors used in the calculations were taken from tables given by Peters and Van Slyke (1932). In all cases the amount of physically dissolved oxygen was calculated using a solubility coefficient of 0.036 and subtracted so that the values given for oxygen represent the quantities combined with hemoglobin.

EXPERIMENTAL RESULTS

Oxygen Capacities and Red Cell Volumes

The upper limit of the quantity of oxygen which a given volume of blood can transport is determined by the oxygen capacity of the blood.

By oxygen capacity is meant the amount of oxygen (expressed as volumes per cent) combined with hemoglobin of whole blood when the pressure of oxygen is 153 mm. (that of air) and when the pressure of CO_2, as shown by analysis of the gas phase, is less than 2 mm. Hg.

Individuals of all four species showed considerable variation in the oxygen capacity. The differences in the mean values for the four species as compared with the individual variations are small and hence the differences in the mean values are of no apparent significance. In comparing the oxygen capacities listed in Table I with the oxygen capacities of various animals compiled by Redfield (1933), the average value for the catfish, carp, bowfin and common sucker is higher than that for most marine fish but is well below the average for mammals.

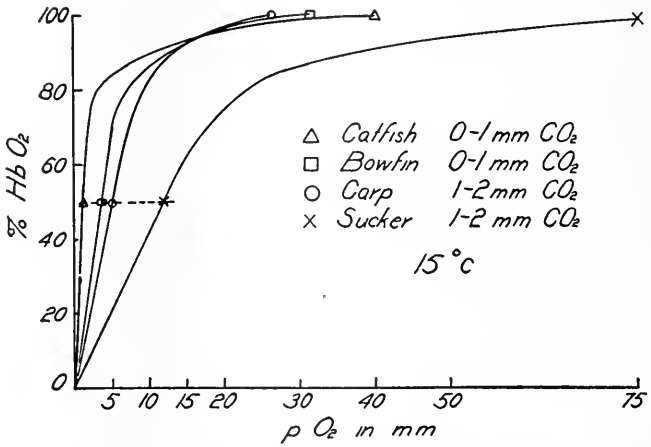

FIG. 1. Oxygen dissociation curves for the blood of the catfish, carp, bowfin and common sucker at pCO_2 0–2 mm.

It is of some interest to note that the mean oxygen capacity for the bowfin, a ganoid, is about equal to the average of the bony fishes while it is twice that found for elasmobranchs by Dill, Edwards and Florkin (1932) and Ferguson, Horvath and Pappenheimer (1938).

Table I also gives the relation of oxygen capacity to red cell volume. Since red cell volume in any one sample of blood varied as a result of conditions not as yet completely investigated, little significance is attached to the values given.

The Combination of Oxygen with Blood

Oxygen Dissociation Curves in the Absence of CO_2.—Because of the metabolism of the nucleated erythrocytes in fish blood it is difficult to obtain oxygen dissociation data in the complete absence of CO_2. In-

asmuch as the effect of pCO_2 2 mm. could not be discriminated from the effect of an indetectable pressure of CO_2 for the blood of the most sensitive fish, the oxygen dissociation curves for all the species at pCO_2 0–2 mm. are considered to be in the virtual absence of CO_2.

Oxygen dissociation curves for the blood of the four species are shown in Fig. 1. The data for the carp are taken from work published previously by Black and Irving (1937); the full data for the other three species are plotted in Fig. 2 (common sucker), Fig. 3 (catfish) and Fig. 4 (bowfin).

The curves for the four species seem to constitute one family of curves rising from the origin without evidence of an inflection which may, however, be present at higher temperatures.

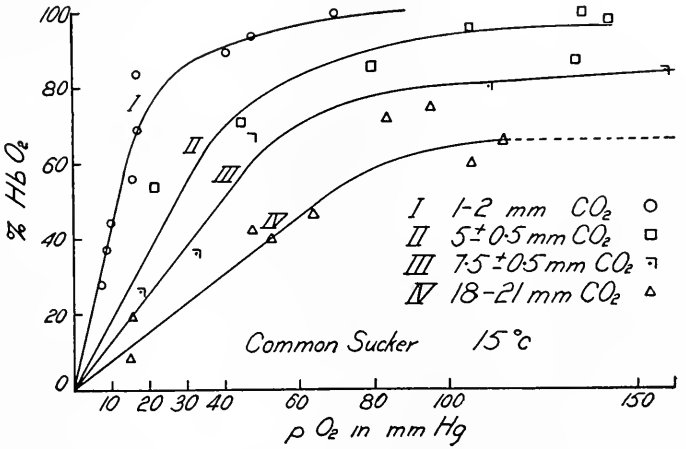

FIG. 2. Oxygen dissociation curves for the blood of the common sucker at various pressures of CO_2. (N. B. Change in scale.)

The pressure of oxygen required to half saturate the blood is often taken as a characteristic of the dissociation curve. Krogh and Leitch (1919) called this the tension of unloading (t_u). This point does not necessarily represent the true conditions for unloading of fish blood so that the pressure at half saturation is used in this paper merely to represent the mid-point of the functional range of the hemoglobin. Further, the pressure of half saturation may be used as a single figure to represent the oxygen affinity of the blood, when the blood is equilibrated in the virtual absence of CO_2. These pressures are given in Table II and reflect the fact that the curves for the catfish, bowfin and carp rise more steeply than that for the common sucker. They also indicate that, under these conditions of CO_2 and temperature, the

oxygen of the blood of the common sucker would be available to the tissues at higher pressures than in the other three fish.

Krogh and Leitch (1919) have also introduced the term "tension of loading" (t_l) to designate the pressure for 95 per cent saturation of

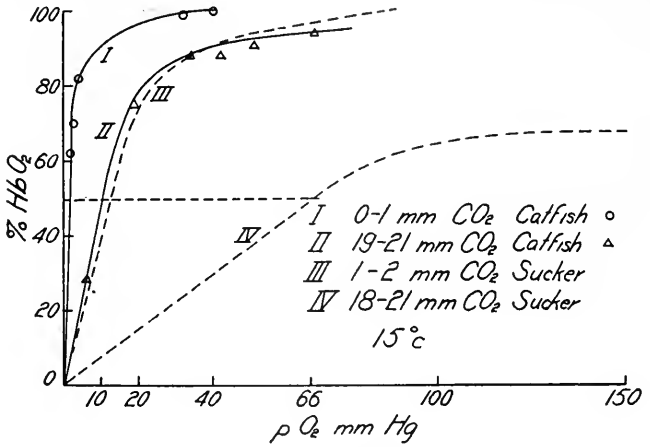

FIG. 3. Oxygen dissociation curves for the blood of the catfish and common sucker at pCO_2 0–2 and 20 mm.

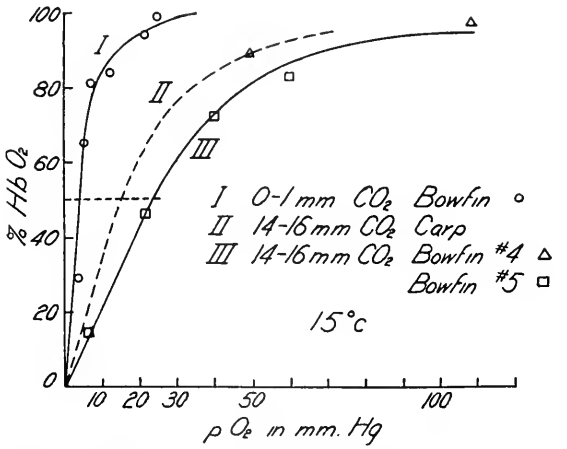

FIG. 4. Oxygen dissociation curves for the blood of the carp at pCO_2 15 mm. and for the blood of the bowfin at 1 and 15 mm. CO_2.

the blood. This is a common degree of saturation for mammalian arterial blood but little satisfactory data exist to show the conditions of the blood coming from the gills of the fish. The loading tensions might also be used to designate the affinity of the hemoglobin for

oxygen and in fact they do in this series correspond qualitatively to the tension of unloading. They are not used here because they cannot be determined with any satisfactory degree of precision owing to the rapidly changing slope of the curves in this region.

The oxygen dissociation curves for blood *equilibrated in the absence of* CO_2 are practically identical for the carp and the bowfin and they

TABLE II

Pressures of oxygen required for half saturation and loading of fish blood at various tensions of carbon dioxide and equilibrated at 15° C.

Species	pCO_2	pO_2 at 50% HbO_2	pO_2 at 95% HbO_2
	mm. Hg	*mm. Hg*	*mm. Hg*
Catfish			
Sample 5 (single)	0–1	1.4	18
Sample 9 (4 pooled)	19–21	10	75
Sample 5 (single)	39	7.5	65
Sample 7 (2 pooled)	41	9	65
Sample 8 (2 pooled)	38	14	85
Carp			
(mean values of plotted data, Black and Irving, 1937)	1–2	5	17
	9–11	8	35
	14–16	14	70
	18–20	20	—
Bowfin			
Sample 4 (single)	0–1	4	19
Sample 5 (single)	12	11	
Sample 5 (single)	15	23	110
Common sucker			
(mean values of plotted data, Fig. 2)	1–2	12	53
	5	27	105
	7.5	40	—
	12*	52	—
	18–21	66	—

* Single point.

stand midway between the widely separated curves for the catfish and the common sucker (Fig. 1). From these data it may be said that the affinity for oxygen of the hemoglobin as it exists in the blood differs significantly in certain species of fish, a generalization first stated by Krogh and Leitch (1919). The marked differences found for the species mentioned above are in contrast to the similarity of the oxygen affinity of the blood of the five marine fish studied by Root (1931) and Green and Root (1933).

The Effect of CO_2 upon the Affinity for Oxygen of Whole Blood Studied

Common Sucker.—It is evident that CO_2 not only decreased the affinity for oxygen in the sense of increasing the pressure of oxygen required for half saturation, but also lowered the maximal amount of oxygen combined, *i.e.* at 150 mm. oxygen, as found by Root for certain marine teleosts. It has been demonstrated on carp and rainbow trout blood by Ferguson and Black (1940) that in the presence of CO_2 even a pressure of 700 mm. oxygen failed to restore the amount of oxygen combined with the hemoglobin to its former value.

It is apparent from Fig. 2 that increasing the pCO_2 from 2 to 20 mm. increases the oxygen pressure at half saturation by about 50 mm. The extreme individual variations found in seven fish give this mean figure a maximal spread of ± 6 mm. of oxygen. The magnitude of the sensitivity to CO_2 of the blood of the common sucker has been further confirmed on blood from nine other fish exposed to tensions of CO_2 of $5 \pm .5$ and $7.5 \pm .5$ mm.

Catfish.—The blood of the catfish showed the least sensitivity to CO_2 both as to decrease of maximal oxygen combined at high oxygen pressures and as to increased pressures of oxygen required at half saturation. Figure 3 contrasts the effect of CO_2 on catfish blood with mean curves of the blood of the common sucker.

The curve plotted for the blood of the catfish at pCO_2 20 mm. represents the sample most sensitive to CO_2 but it shows an increase of only 8 mm. in the pressure of oxygen at half saturation and is in sharp contrast to the mean figure of 50 mm. for the common sucker. The difference between these species in sensitivity to CO_2 is far beyond the range of individual variation.

Carp and Bowfin.—Data for carp and bowfin are plotted in Fig. 4. The CO_2 sensitivities of the blood of these two species do not appear to differ greatly. The sensitivities are between those of the common sucker and the catfish but much closer to the latter. Very extensive studies would be necessary to establish a significant species difference between the carp and the bowfin though it may well exist.

Discussion

Von Ledebur (1939) cites Hall (1929) and Root (1931) and concludes that in certain instances, especially among marine fish, there seems to be a parallelism between the oxygen affinity of the hemoglobin and the activity of the species. However, Hall (1929) stated that in the three species studied the correlation was with the *amount* of hemoglobin per unit volume of blood, not with oxygen *affinity* of the hemoglobin. Root (1931) stated that "sluggish fish have bloods of low oxygen

capacity and the active of high capacity." Our data on oxygen capacities allow no deductions on this point since significant species differences were not found.

The differences in oxygen affinity are quite striking. An examination of Fig. 1 shows that the blood of the carp could be oxygenated to the extent of 90 per cent at a pressure of oxygen of 12 mm., at which pressure the blood of the common sucker is only half saturated. Again the remarkable blood of the catfish could serve at an environmental oxygen tension quite unsuited to the blood of the other species, for at pO_2 3 mm. its blood would be 80 per cent saturated while the blood of the carp and common sucker would only be 35 and 12 per cent oxygenated respectively.

Krogh and Leitch (1919) suggested a relation between the affinity of the blood for oxygen with the ability of the species to survive in environments low in oxygen. That this is an important relation is supported by the work of Fry and Black (1938), who have shown that the catfish, *A. nebulosus*, was able to remove oxygen at a lower tension than any of the other fish among the large series of fishes studied; as Wells (1913) found to be the case for a related species of catfish, *A. melas*.

The significance of the species difference in CO_2 sensitivity of the blood in relation to the ability of the blood to transport oxygen may be seen by considering the following hypothetical situations. If blood be fully oxygenated at the gills in the absence of CO_2 and transported to the tissues where metabolism proceeds until the pressure of both CO_2 and oxygen is 20 mm., then 25 per cent of the combined oxygen could be discharged from the blood of the catfish, 50 per cent from that of the carp and 85 per cent from the blood of the common sucker. Or viewed in another manner, the blood of the common sucker can be loaded (95 per cent HbO_2) at a pressure of 53 mm. Were this blood now exposed to the tissues where a pressure of 20 mm. CO_2 obtained, then 55 per cent (from 95 to 40 volumes per cent) of the combined oxygen could be utilized without any loss in the pressure of oxygen first required for loading. Under similar circumstances only about 15 per cent (from 95 to 80 volumes per cent) of the combined oxygen could be discharged by the blood of the catfish at its loading pressure of about 18 mm.

In terms of pressure of oxygen produced (at constant oxygenation) the specific differences in the ability of CO_2 to affect the equilibrium between oxygen and whole blood are no less interesting. If blood first oxygenated at the gills in the absence of CO_2 be transferred to the tissues where a tension of 20 mm. CO_2 exists, then when one-third

of the combined oxygen is used, the pressures of oxygen available to the tissues will be of the order of 15 mm. in the catfish, 30 mm. for the carp and 115 mm. for the common sucker.

It is an accepted concept of mammalian physiology that the Bohr effect contributes significantly to the efficiency of oxygen transport and might under certain circumstances be an essential factor in transportation of oxygen. Consequently it was thought that the wide differences in CO_2 sensitivity might reflect differences in habitual activity or capacity for maximal activity. Unfortunately, little data are available on the activity of fishes, habitual or maximal, in their natural environment or on metabolism, basal or maximal.

If CO_2 sensitivity of the blood is related to activity one would espect that the maximal activity of the catfish would be of a very low order. However, the maximal oxygen consumption of actively swimming catfish was found to be greater than the maximal oxygen utilization of the perch and common sunfish (Black, Fry and Scott, 1939). The blood of the perch is much more sensitive to CO_2 than the blood of the catfish (Irving, Black and Safford, 1939). It would seem then that no correlation between the CO_2 sensitivity of the blood and the rate of oxygen uptake of actively swimming fish is apparent. From this the inference is made that while the enlarged Bohr effect must be of importance to the efficiency of oxygen transport, each of the other factors such as circulatory efficiency, environmental temperature, the tension at which tissues can utilize oxygen, etc. is so important that the existence of a correlation between the activity of the fish and the single effect of CO_2 upon oxygen affinity of the hemoglobin may be masked. For example, J. S. Hart (1940) in his estimation of cardiac output for the catfish, carp, bowfin and common sucker found that the stroke volume of the catfish greatly exceeded the others while the heart of the common sucker had the smallest output per beat.

The possibility that the CO_2 effect upon the oxygen affinity may function as an oxygen pump is of the utmost interest in connection with the swimbladder. In their efforts to determine whether the mammalian lung secreted oxygen, Bohr and Haldane were greatly concerned with the mechanism for the deposition of gases in the swimbladder. An inspection of the oxygen dissociation curves of the blood of the common sucker at various pressures of CO_2 shows that the pressure of oxygen could be greatly increased by small pressures of CO_2. These data extend the quantitative basis for the theory as put forth by Haldane (1922), Hall (1924) and Jacobs (1930). Of particular significance is the experimental demonstration (mentioned in results) that CO_2 will dissociate oxygen from hemoglobin at pressures up to 700 mm. Hg.

While the increased sensitivity to CO_2 should enhance the use of the blood in transporting oxygen and in making available a higher pressure of oxygen at unloading, this very sensitivity should hinder oxygenation at the gills. Fry and Black (1938) tested this implication and found that the common sucker with its CO_2-sensitive blood was unable to remove oxygen from water containing CO_2 at tensions which did not hinder the respiration of catfish. Catfish proved to be the most resistant to the presence of CO_2 of all species studied, and it is a well-known fact that catfish are often found in swamps of acid water.

Although there is little evidence of significant differences in oxygen capacity, the experiments suggest strongly that the differences (a) in oxygen affinity of the blood and (b) sensitivity of the oxygen affinity to CO_2, are important in natural respiration. Krogh and Leitch suggested that species variations might be considered as adaptations to environmental conditions, specifying particularly the degree of saturation of the water with oxygen. The experiments reported show that, in the catfish a high oxygen affinity is associated with low sensitivity to CO_2 while in the common sucker a low oxygen affinity is associated with a high CO_2 sensitivity. If specific differences in oxygen affinity constitute a basis for adaptation, then differences in CO_2 sensitivity of the blood must be considered as part of the adaptation of the blood. Krogh and Leitch recognized the importance of the CO_2 effect but did not observe that it differed greatly among the species.

The possession of blood characterized by a high affinity for oxygen would be expected to allow utilization of oxygen from water in which it was dissolved at a low tension. Such a hemoglobin, however, would impose the disadvantage that this oxygen would only be available to the tissues at a low pressure. This disadvantage might be offset by a high sensitivity to CO_2 whereby the CO_2 in the tissues would release the oxygen at a higher pressure. However, these experiments show that the catfish which has the highest oxygen affinity has no great CO_2 effect to offset it but indeed has the smallest CO_2 effect of the fish studied.

One other factor which might offset the apparent disadvantage of high oxygen affinity would be a higher environmental temperature, for higher temperature, like CO_2, also tends to lower oxygen affinity or raise the pressure of unloading (t_u). It appears that the catfish in the summer frequents the shallower warmer water (Fry, 1939) and it is now apparent how the characteristics of the blood of the catfish should be more suitable in the warmer than in cooler strata of water.

The common sucker is found in deeper water in summer (Fry, 1939) where the possession of lower oxygen affinity and higher CO_2 sensitivity

offset the tendency of the lower temperature to decrease the pressure of unloading (t_u).

Experiments of Irving, Black and Safford (1940) (done at 15° C.) have shown that three species of trout (which are typically found in cold water), possess blood of even lower affinity for oxygen and greater sensitivity to CO_2 than that of the common sucker.

The low sensitivity to CO_2 and the high affinity for oxygen of the blood of the catfish are properties which could best be utilized for the transport of oxygen at higher rather than lower environmental temperatures. Conversely a low oxygen affinity and a high CO_2 sensitivity of the blood (such as characterize that of the common sucker or the trout) are properties which would tend to offset the lowered temperature effect in the transport of oxygen by the blood. The separate adaptive features of lowered oxygen affinity and enlarged Bohr effect of fish blood in an environment of low temperature were set forth by Krogh and Leitch (1919). However, their data did not show that high affinity for oxygen is in general possessed by blood of low CO_2 sensitivity and that blood of low affinity for oxygen has a large CO_2 effect. Moreover, their data did not show that species differences existed in the sensitivity of the blood to CO_2. That gradations in the CO_2 effect may be of importance in the economy of fish inhabiting different thermal strata of freshwater lakes has been discussed very clearly by Fry (1939).

Krogh and Leitch suggested that the specific characteristics of the hemoglobin in the different bloods must be due to specific conditions in the different erythrocytes. This view was based on the belief common at that time that the hemoglobin was identical in all species. It is now known that considerable differences in the properties can be found due to differences in the globin. It remains to be shown to what extent the specific characteristics of these bloods are due to different hemoglobins and to what extent due to special conditions in the erythrocytes. Hall and McCutcheon (1938) have made an extensive examination of the differences in the hemoglobins of marine fish. It seems important to point out that these differences in hemoglobins do not always explain the sum total of the differences between whole bloods as regards oxygen transport, for Black and Irving (1938) have shown for the blood of the carp, bowfin and common sucker that when hemoglobin is released by hemolysis the effects of CO_2 on oxygen affinity become so strikingly different that it is at present impossible to predict the properties of the hemoglobin within the red cell from its observed properties in solution. The explanation given by Krogh and Leitch for the basis of adaptation in the blood of fish

may then be extended, for the specificity of the oxygen affinity of the hemoglobin and the sensitivity of the hemoglobin to CO_2 as it exists in fish blood are undoubtedly a combination of the two factors, specificity of the hemoglobin moiety and the specific influence of the intact corpuscle.

Summary

1. Oxygen capacities have been determined on the blood of four freshwater fish (catfish, carp, bowfin and common sucker). Individual variations are larger than average interspecific differences.

2. Significant differences between species were found for the affinity of the hemoglobin in whole blood for oxygen in the absence of CO_2 and at 15° C. in these freshwater fish as contrasted with the rather uniform oxygen affinity of most marine fish studied so far.

3. Significant differences between species were found for the effect of CO_2 on the affinity of the hemoglobin in whole blood for oxygen. For example, at 15° C. an increase in pCO_2 from 2 to 20 mm. causes an increase in oxygen pressure at half saturation of 8 mm. for the catfish and of 50 mm. for the common sucker.

4. Blood with high oxygen affinity has a low sensitivity to CO_2 (catfish). Blood with low affinity for oxygen has a high CO_2 sensitivity (common sucker).

5. It is suggested that since blood characterized by high oxygen affinity lacks a large CO_2 effect which could offset the disadvantage imposed at deoxygenation, the blood would function best in a high environmental temperature.

6. The greater sensitivity to CO_2 of fish blood as compared with mammalian blood was regarded by Krogh and Leitch as an adaptation to lower temperature. Evidence can now be presented to show that freshwater fish of higher CO_2 sensitivities are found in the colder habitats.

7. The very striking effects of CO_2 show that high pressures of oxygen might be developed within the fish by the presence of relatively low pressures of CO_2. The old suggestion that the Bohr effect may be responsible for the high percentage of oxygen in the swimbladder of many fish is thus given additional quantitative support.

Acknowledgments

It is a pleasure to acknowledge my indebtedness to Professor Laurence Irving, under whose direction this study was made. From the outset he has been most liberal not only with his advice but also with his criticism and encouragement.

It would have been impractical to carry out this study had it not been for the kindness of those who made available fresh supplies of live fish. I refer to Mr. S. J. Bowman of Toronto, Mr. A. Bralow of Philadelphia and Mr. C. R. Buller, Chief Fish Culturist of the Pennsylvania Fish and Game Commission. I am also obligated to Messrs. Alfred Quantrill and Stephen Clare of Toronto and to Mr. Otto Hebel of Swarthmore for their unstinting and invaluable technical assistance.

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THE

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DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER[1]

III. Influence of Temperature and Source and Condition of Water

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(*From the Woods Hole Oceanographic Institution, Woods Hole, Mass.*)

The first two papers in this series (1937, 1939) have shown that the decomposition of suspended organic matter is accompanied by the successive formation of ammonia, nitrite and nitrate in the sea water medium in distinctly recognizable stages; that fresh diatoms can grow in the water at any stage in the cycle; and that successive cycles of decomposition and regeneration can be carried out in the same water, without the addition of new organic substrate. Since the analogous processes in the sea can scarcely be expected to take place in such simple, successive fashion, it is important to know how the decomposition cycle is influenced in rate or character by a number of variable factors. Among these are temperature, source of the sea water, and the presence or absence of such micro-organisms as can be controlled by the routine technic of sterilization. The present paper describes an experimental study of these factors.

Earlier work had shown that the nature of the decomposition cycle varied somewhat with the source of the water, being generally more rapid in water from the harbor than in that from offshore. To pursue this point further three samples of water were taken from a deep-sea station (25°–32′ N.; 53°–45′ W.): one from the surface, one from the level of the oxygen minimum (800 meters), and one from 1200 meters. Each of these samples was divided into two parts, one of which was "sterilized" by suction filtration through a No. 5 sintered-glass funnel. There were previous indications that this treatment was adequate to remove bacteria.

To each of the six portions of sea water (about two liters each) were added equal amounts of washed *Nitzschia Closterium,* which, as in

[1] Contribution No. 268 from the Woods Hole Oceanographic Institution.

previous experiments, served as a convenient source of organic matter. The suspensions were placed in the dark, at laboratory temperature, and periodically analyzed for particulate nitrogen, ammonia, nitrite and nitrate, in the manner already described. The results are shown in Fig. 1.

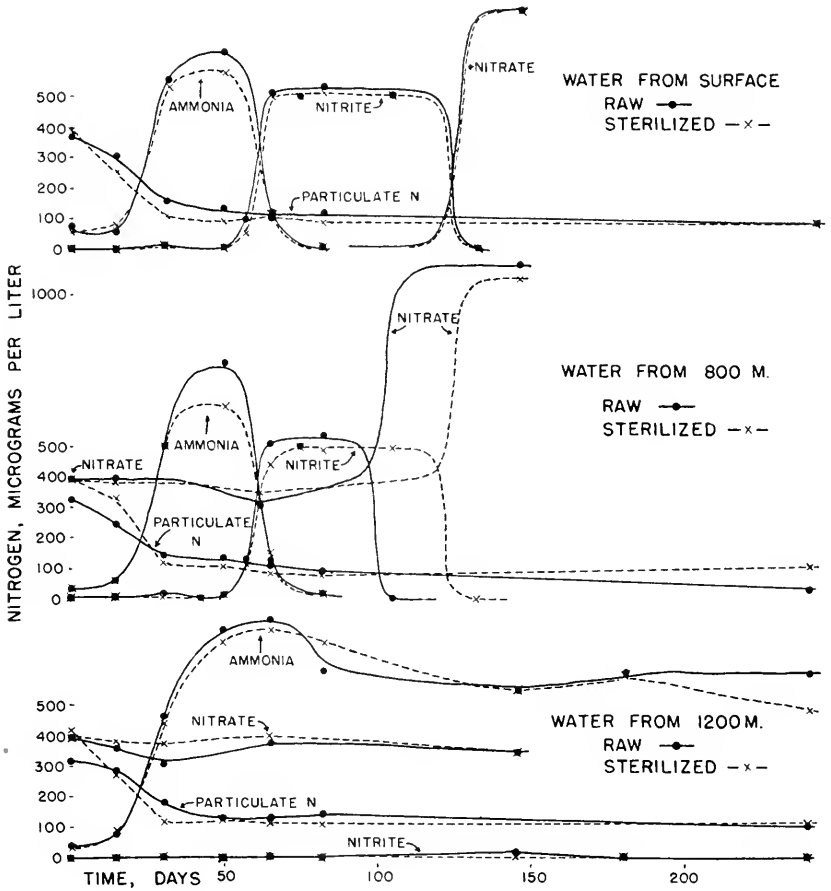

FIG. 1. The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate. Fresh cultures of *Nitzschia Closterium* suspended in sea water from different depths, part of which has been sterilized by filtration.

There was no difference in the behavior of those samples which had been sterilized by filtration and those which had not. The decomposition proceeded in the normal manner in the surface water and in that from the oxygen minimum layer. But in the deep water (1200 m.)

there was no oxidation of ammonia during the time of the experiment (250 days). The nature of this inhibiting effect is yet to be determined. That it is not due simply to the absence of bacteria seems evident from the fact that such oxidation did proceed in the other samples, whether or not the water had been sterilized. This would indicate that the necessary organisms were carried by the diatoms. But since the same diatom culture was used in the deep water, the failure of oxidation to take place is evidently due to some other, unexplained factor. It should be pointed out that ammonia and nitrite are seldom found below 200 or 300 meters in the sea.

Since the oxidizing organisms seemed to be carried by the diatoms rather than in the water itself, another series of experiments was carried out in which harbor water was used as the suspension medium, part of which was sterilized by filtration, as before. To portions of the sterile and the raw water washed diatoms were added, as in the last series. To other similar portions of the water (sterile and raw) were added equal amounts of a diatom suspension which had been boiled for two minutes to sterilize it. Precautions were taken to insure the addition of equal amounts of organic matter to all four of the cultures, after which they were placed in the dark and sampled periodically, according to the usual routine. The results are shown in Fig. 2.

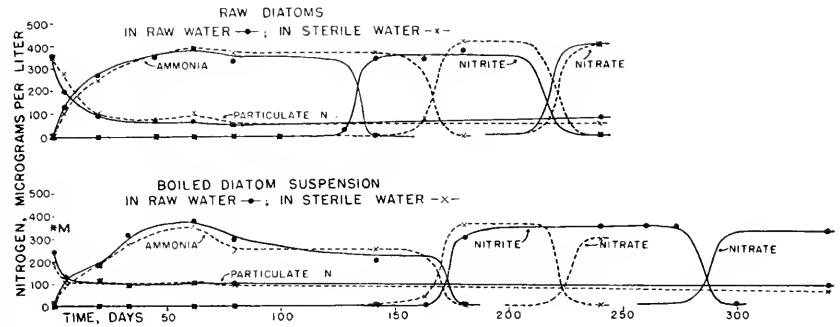

FIG. 2. The disappearance of particulate nitrogen and the simultaneous changes in ammonia, nitrite and nitrate, in harbor water, one portion sterilized by filtration. Upper curves: fresh cultures of *Nitzschia Closterium*. Lower curves: *Nitzschia* cultures boiled to sterilize. The point marked *M indicates the amount of particulate nitrogen in the *Nitzschia* culture before boiling.

The boiling of the diatom suspension quite evidently throws some of the organic matter into solution, for, although equal quantities of diatoms were used, the first determination of particulate nitrogen is much lower in the cultures to which the boiled suspensions were added than in those containing the same amount of raw diatom material. The

boiling of the diatoms also resulted in a somewhat slower rate of ammonia formation and a prolongation of the ammonia stage of the cycle.

The fact that the decomposition cycles were so nearly alike in the sterilized and the raw sea water, and that oxidation of ammonia to nitrite and nitrate took place in all cases, raises the serious question whether the filtration had actually sterilized the sea water, or whether the cultures had been contaminated with nitrite-forming organisms from the air. It is difficult to explain the formation of nitrite and nitrate except upon one of these two assumptions and we are not as yet able to resolve this difficulty. The culture methods for the identification of nitrite-forming bacteria are so unsatisfactory that we did not attempt to use them upon our material. It is almost impossible to use aseptic technic in sampling the cultures, although the chance of contamination can be diminished by pouring rather than pipetting samples.

In the effort to get further information on this point, four supposedly bacteria-free cultures of *Nitzschia* were obtained. One of these was analyzed immediately and found to be free of ammonia, nitrite and nitrate. The others were placed in the dark and analyzed later. After 145 days one culture contained 500 γ of ammonia nitrogen and 10 γ of nitrite nitrogen per liter. After 300 days the second contained 870 γ of ammonia nitrogen and 35 γ of nitrite. A large amount of suspended material still remained in both cases, but it was evident that considerable decomposition had taken place.

The last culture was opened and large numbers of bacteria were found in the remaining suspended matter. Even with great care we had not succeeded in preparing and preserving sterile cultures. Nevertheless, it seems worthwhile to note that bacteria developed only very slowly and that an almost negligible amount of ammonia had been oxidized to nitrite in 300 days. Apparently it is easier to eliminate the organisms responsible for oxidation than those which may possibly take part in the formation of ammonia.

To determine the temperature coefficient of the decomposition process, three suspensions of washed cells of *Nitzschia* were prepared in the usual manner. These were then placed in the dark at constant temperatures: one at 15° C., one at 7–9° C., and one at 1–2° C. By a stroke of good luck, when the first complete analysis was made, after 280 days, the one at the lowest temperature (No. 28) was in the ammonia stage, the one at the middle temperature (No. 29) was in the nitrite stage, and the one at the highest temperature (No. 30) had completed the cycle and all the nitrogen was in the form of nitrate. The complete results are given in Table I.

Unfortunately, No. 29 was lost at about the time when the nitrite had disappeared, but the cycle was so nearly completed at the time of the last preceding analysis that a sufficiently accurate estimate of the total elapsed time could be made. At the date of writing, after 635 days, No. 28 had not gone beyond the ammonia stage.

TABLE I

Series Nos. 28, 29 and 30. Sea water containing fresh cultures of *Nitzschia Closterium* kept in the dark at different temperatures as shown. Micrograms of nitrogen per liter.

	Date	Particulate	Ammonia	Nitrite	Nitrate
No. 28	9–14–38	470	10	0	10
Temperature	10– 3–38	260			
1 to 2° C.	1– 6–39	244			
	4–17–39	98			
	6–22–39	134	385	0	15
	8–30–39	64			
	2–26–40	24	310	0	10
	6–11–40	24	385	0	5
No. 29	9–14–38	470	10	0	10
Temperature	10– 3–38	129			
7 to 9° C.	1– 6–39	75			
	4–17–39	51			
	6–22–39	67	21	330	10
	8–30–39	41	20	320	
	9– 7–39			200	
	2–26–40	25	0	45	
No. 30	9–14–38	483	5	0	10
Temperature	10– 3–38	38			
15° C.	1– 6–39	49			
	4– 7–39	47			
	6–22–39	57	18	0	375
	8–30–39	28	25	0	400
	2–26–40	40	0	0	400
	6–11–40	18	0	0	400

The complete cycle in No. 30 (15°) took less than 280 days, and in No. 29 (7–9°) about 550 days. A temperature difference of 6 to 8° C. therefore more than doubled the velocity of the over-all process. Since more than one reaction mechanism is involved in the cycle, the relation is probably not a simple one, and it is indeed possible that a certain minimum temperature is necessary for the oxidation of ammonia.

On September 2, 1939, portions of cultures Nos. 28, 29 and 30 were inoculated with fresh diatoms and placed in the light. At this time the maximum amounts of ammonia, nitrite and nitrate, respectively, were

in these three cultures. Two weeks later there was no ammonia, nitrite or nitrate in any of the three, and an abundant diatom growth was observed. This confirms definitely our earlier observation that new diatoms can grow in the medium at any stage in the cycle of decomposition.

Summary

The source of the water is important in determining the nature of the decomposition cycle. Oxidation of ammonia to nitrite is retarded in water from the deep sea (1200 m.). Inconclusive results were obtained from efforts to sterilize both the water and the original organic matter, but it is evidently easier to eliminate organisms responsible for the oxidation processes than those which may participate in the formation of ammonia. The speed of the whole decomposition cycle was more than doubled by an increase of 6° or 8° in temperature. Growth of diatoms is possible at any stage in the cycle of decomposition.

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THE CHROMATOPHORE SYSTEM IN THE CATFISH AMEIURUS

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Introduction

The color changes in the catfish, *Ameiurus nebulosus,* have attracted general attention for at least two decades, but they have been studied in detail only during the last five or six years. As receptors for such changes the eyes in this fish have been recognized by all workers. In 1917 Van Heusen showed that the innervation of the catfish skin was photosensitive and some two years ago Wykes (1938) pointed out that this sensitivity may play a part in the color changes in this fish. The pineal organ has also been suspected of influencing the chromatic activities in *Ameiurus* and the direct stimulation of chromatophores by light has been invoked by some workers to this end.

The chromatophores in *Ameiurus* appear to be all melanophores, micromelanophores in the epidermis and macromelanophores directly under the derma. Of these the macromelanophores are much the more convenient for study. The melanophores in *Ameiurus* appear to be activated through three sets of neurohumors, intermedin from the pituitary gland, acetylcholine from the dispersing autonomic nerve-fibers and adrenaline from the concentrating fibers. The first two neurohumors disperse the melanophore pigment and thereby darken the fish; the third concentrates it and thus induces blanching (Parker, 1940*a*). The object of this paper is to discuss the systematic relations of catfish melanophores, their neurohumors, and the receptors concerned with their activation.

Specific Responses of Melanophores

The melanophores of *Ameiurus* respond very differently depending upon the particular conditions of the fish: presence or absence of eyes, of pituitary gland, whether the fish is in darkness or in light, and the nature of the background white or black. Many of these types of response have been recorded already by students of this subject some of whom have partly tabulated them (Abramowitz, 1936; Osborn, 1938*b*).

Table I of the present paper summarizes my own observations on the tint of the tail of *Ameiurus,* which is the same as that of the body generally, as well as on the tint of newly cut caudal bands under different conditions. These conditions, of which sixteen are recorded, are indicated on the left-hand side of Table I.

Table I

Tints of the tails and of the newly cut caudal bands of the catfish *Ameiurus* under various conditions: with or without eyes, with or without pituitary glands, in light on white or black backgrounds, and in darkness as pale fishes or dark ones. The scale of tints in relation to the average diameters in microns of the macromelanophores is approximately as follows: pale, 45; palish, 60; intermediate 70; darkish, 95; dark, 138; coal-black, 143.

Conditions of fishes		Tints: Tail	Tints: New band
Eyed			
With pituitary gland			
In light			
White background	1	Pale	Darkish
Black background	2	Dark	Coal-black
In darkness			
Pale fish	3	Pale	Darkish
Dark fish	4	Dark	Coal-black
Without pituitary gland			
In light			
White background	5	Palish	Darkish
Black background	6	Intermediate	Darkish
In darkness			
Pale fish	7	Palish	Darkish
Intermediate fish	8	Intermediate	Darkish
Eyeless			
With pituitary gland			
In light			
White background	9	Coal-black	Coal-black
Black background	10	Coal-black	Coal-black
In darkness			
Pale fish	11	Pale	Darkish
Dark fish	12	Dark	Coal-black
Without pituitary gland			
In light			
White background	13	Intermediate	Darkish
Black background	14	Intermediate	Darkish
In darkness			
Pale fish	15	Palish	Darkish
Intermediate fish	16	Intermediate	Darkish

The diameters of the pigmented areas of the macromelanophores in *Ameiurus* are about 40 microns in the concentrated state and 145 microns in the dispersed one and of the micromelanophores about 12 and 90 microns respectively. Wykes (1938) and Osborn (1938*b*) in their

studies of the catfish color changes have measured the diameters of considerable numbers of melanophores under different conditions and expressed their results in averages. My own procedure has been somewhat different. I have preferred to use adjectives as descriptive of the states of the color cells instead of cell measurements and I have distinguished in the scale of changes six steps from extreme dispersion of pigment to its extreme concentration (Table I). These steps have been judged in any given fish by the eye in comparison with the tints of other fishes kept as standards. This can be illustrated by the most difficult steps to distinguish that between dark and coal-black.

To carry out this separation standards were made in the following way. Two catfishes closely matched in color were kept in an illuminated, black-walled aquarium for several days till they had come to a uniform deep tint, dark. The eyes of one of these fishes were then removed whereupon the fish, still illuminated, quickly deepened in tint to what has been called coal-black. In the illuminated, black-walled aquarium these two fishes, one dark and the other coal-black, continued to maintain with great persistence these two tints. They could then be used as standards for estimating the colors of other experimental fishes. By appropriate steps additional fishes were standardized for other points in the scale and the fishes thus prepared were used in judging the color of fishes under test. In my opinion this method is a more delicate and certain one for determining tints than that of measuring macromelanophores.

Another feature in the measurement of melanophores should be mentioned. Catfishes of the lengths used in these studies, about 15 cm., are continually losing melanophores and forming new ones (Odiorne, 1937). Small macromelanophores are therefore presumably young ones and in process of increasing in size. As a means of comparison in the present study a considerable number of melanophores under a variety of conditions were measured, but in consequence of the way in which melanophores grow these measurements were made not on the total population of color cells but on the large and presumably mature cells. This selective process is reasonable, for what is of concern at this stage is not the diameters of melanophores in general, but the diameters of their pigment areas as seen in their dispersed and their concentrated states.

On several occasions during these studies full sets of melanophore measurements were carried through on all the conditions of catfishes noted in Table I. These consisted of micrometer eye-piece measurements on at least ten mature macromelanophores in each of three catfishes for all of the sixteen states given in the table. These measure-

ments afforded the basis for the rough estimates of the diameters of macromelanophores in relation to the tints of the fishes as stated in the heading for Table I. In general they are in line with the kind of measurements made by Wykes and by Osborn. Of these steps in color changes the one from dark to coal-black has long been known and recognized as of considerable importance. Yet its difference in terms of melanophore diameters, about five microns, is scarcely within measurable limits though it is easily noted by direct inspection. Hence, as already stated, inspection by eye and the use of standard fishes may be a more effective way of discovering color differences than measuring melanophores.

No detailed comments as to the contents of Table I will be made here, but the data in this table will be referred to frequently in the course of the following discussion. A few general remarks, however, may not be inappropriate. The fully pale fishes are those with eyes, pituitary glands, in bright light and on a white background (Table I, 1). This high degree of paleness disappears with the loss of the pituitary gland (5). The coal-black fishes are eyeless, but with pituitary glands and in the light (9, 10). Newly cut bands are coal-black only when induced in fishes which have not been hypophysectomized (2, 4, 9, 10, 12). No hypophysectomized fish either in the tint of its tail or in that of its band ever reaches full paleness or coal-blackness (5 to 8 and 13 to 16). These limitations have already been pointed out by Osborn (1938*a*), Veil (1938), Wykes (1938), Enami (1939), and Parker (1940*a*). The states in darkness recorded in the table involve resting states of melanophores (Parker, 1939; 1940*b*) and hence fishes showing the two possible extremes of tint at the time of their introduction into darkness are given, pale or dark for those with pituitary glands (3, 4, 11, 12) and palish or intermediate for those without these glands (7, 8, 15, 16).

The Activation of Melanophores

The activation of the melanophores in *Ameiurus* has been attributed to the direct action of light on these color cells or by means of such receptors as the pineal organ, integumentary photoreceptors, and the eyes. The direct action of light on the melanophores of the catfish was suggested as early as 1934 by Parker. It was tested experimentally by Wykes (1938) who found that a narrow beam of light focused on the skin of a catfish by means of a lens was not followed by any chromatophoral response. I have also tested this matter experimentally. The tail of a catfish of intermediate tint was split longitudinally into halves by an incision running from the edge of the tail to its root and parallel

with its rays. A fish with a tail thus split was tied on its side to a black board and provided with a current of fresh water through its mouth and gills for respiration. The split tail was then pinned out on the board with one half of it freely exposed and the other half covered with a light-proof, metal chamber so fitted into the board and about the tail that the portion of the tail within it was entirely inaccessible to light. The whole preparation was set up in a dark room and the exposed half of the tail was illuminated by a beam of light from an otherwise completely obscured electric lamp. Thus on the same fish one half of the tail was brightly illuminated and the other half was in full darkness. The fish, which remained normally alive and active under these conditions, was held in this position for a little over an hour. Its tail was then cut off, immediately fixed expanded in hot water at 60° C. and prepared for microscopic examination. In all, three such preparations were made. To the unaided eye the two halves of the tail in each of these were indistinguishable in tint and a microscopic examination of their melanophores showed no consistent differences. I therefore agree with Abramowitz (1936) and with Wykes (1938) that there is thus far no evidence in favor of the direct response of the melanophores in *Ameiurus* to light and I regard this possible factor as negligible in dealing with the color changes in this fish.

Since the appearance of papers by von Frisch (1911) and by Scharrer (1928) the pineal organ has been an object of interest to students of the color changes in the lower vertebrates. Wykes (1938) opened the cranium of *Ameiurus* from the dorsal side and destroyed the pineal organ and a part of the diencephalon with a hot needle, but found that after the fish's recovery its color responses to light persisted normally. She pointed out that the roof of the skull and the skin in *Ameiurus* were so dense that the brain seemed well protected from light. She therefore doubted if the pineal organ in *Ameiurus* was functionally concerned with color changes.

Although the roof of the cranium of the catfishes with which I worked was very dense, as had been remarked by Wykes for her own material, this part of the catfish is by no means impervious to light, as can be seen by holding a preparation of the roof of the head between one's eye and an electric light. To prepare catfishes for pineal tests I cut the integumentary nerves to the top of the head to eliminate the possible action of photoreceptors in the skin of that part and then loosened the skin over the head so that a light-proof, thin, metal sheet could be slipped under the skin to cover fully the pineal region. Such fishes were then subjected to bright local illumination over the pineal organ with and without the light-proof sheet in place. In none of these tests did I

notice any change in tint in the experimental fish as compared with a second fish bound next to the one tested and used as a control. I therefore conclude that however the pineal organ may function in the color changes of other fishes, it gives no evidence in *Ameiurus* of being concerned with chromatic responses. My opinion is thus in agreement with that of Wykes.

It will be remembered that some years ago Kropp (1929) pointed out that aqueous extracts of the eye-balls of dark-adapted tadpoles of *Rana clamitans* yielded a mixture that when injected into other tadpoles induced an expansion of their melanophores. To test this possibility in the catfish I ground to a pulp in a mortar with sand and 2 cc. of Ringer's solution ten pairs of eyes from dark catfishes, extracted the juice and injected 0.5 cc. of this juice into a pale catfish and an equal amount into a dark one. I also made a similar extract from the eyes of ten pale catfishes and injected equal volumes of this extract into a pale and into a dark fish. Careful inspection, however, showed no change whatever in the colors of the recipients either local or general and I was forced to conclude that the eye-balls of *Ameiurus* were probably not concerned in any way as centers for the production of hormones of significance in color changes.

This negative evidence from direct stimulation, the activity of the pineal organ, and of the eye-balls as secretory centers is in strong contrast with the positive results from the two other means of activation, the eyes and the integumentary photoreceptors. All workers on the color changes of catfishes have recognized the great importance of the eyes in these responses. Moreover, the necessary presence of another type of receptor, such as the integumentary photoreceptor, has been repeatedly emphasized (Abramowitz, 1936; Matsushita, 1938; Wykes, 1938). To distinguish in an experimental way the parts played by the eyes and the skin receptors in *Ameiurus* is by no means easy. One operational procedure to this end has been found possible. It consists in a complete transverse section of the brain of the catfish in the region of the cerebellum. When such a section is made the portion of the brain anterior to it carries the eyes, the pituitary gland and their connections undisturbed and no other organs of significance for color changes, for the only other components connected with this part of the brain are, in addition to the pineal organ just discussed, the olfactory nerves and the nerves to the eye-muscles. The portion of the central nervous organs posterior to the cut, the hind part of the brain and the whole of the spinal cord, has attached to it all the cutaneous nerves, from the trigeminal to the last spinal. Thus this cut separates completely and perfectly the eyes and their immediate central connections from the nerves

to the whole of the skin and their immediate centers. Such a preparation has its limitations, but is of great importance in determining the rôles played by the two chief systems of chromatic receptors at present under consideration.

The operation for the transverse severance of the brain of the catfish is carried out without great difficulty. The cut is made in a plane transverse to the longitudinal axis of the fish and a little posterior to the anterior edges of the pectoral fins. In executing this cut in a catfish a small aperture is bored through the skin and bony roof of the cranium of the fish on the dorsal median line and in the transverse plane already described. A scalpel with a very narrow, long blade is then inserted through this aperture and with one transverse cut to the right supplemented by a second one to the left the brain may be completely severed cross-wise. In the catfish the brain is not always uniform in location in reference to external landmarks. A transverse cut through the brain, however accurately made, may therefore not always fall in the desired region. If the cross-cut is a millimeter or so too far anterior, the whole optic and pituitary organization may be interfered with and the preparation thus rendered worthless. If it is too far posterior, it may disrupt the medulla oblongata and disturb the respiratory centers to such an extent that the fish will quickly die. If, however, the cut is successful, the desired separation in the brain may be satisfactorily accomplished and the fish after a few days will be ready for tests. I was fortunate in this operation in about one in every four or five fishes. This rather heavy loss was due, I believe, to the considerable variation in the position of the brain in relation to the external topography of the fish as already noted. Fishes successfully operated on commonly lived for from three to four weeks. They were quite active, but their swimming was disorganized and their orientation completely lost. These motor disturbances were doubtless due to the partial destruction of the cerebellum. Such fishes were, however, well adapted as material for chromatic tests.

A pale catfish, when operated upon as already described, will remain pale apparently indefinitely even in a black-walled, illuminated aquarium. One of my fishes lived thus for 24 days after its brain had been transected and died finally because of further operations. As might be supposed, these fishes remain indefinitely pale on illuminated, white backgrounds. Since, however, they stay pale in black, illuminated surroundings and with their eyes, pituitary glands and the connections of these parts fully intact, one is justified in concluding that the eyes of the catfish are not the receptors concerned with the discharge of intermedin from the pituitary gland.

Dark fishes with brains transected through the cerebellum remain dark irrespective of their surroundings and in their unchangeableness resemble pale fishes. The brain transection, in fact, appears to reduce both kinds of fishes to a stage of color immobility. They are in truth, as I have elsewhere shown (Parker, 1940*b*), in a state of chromatic rest. This brain operation appears to render the whole melanophore system of the catfish inoperative. That the melanophores of such fishes are not injured by the transection but are merely rendered quiescent can be shown by the injection of intermedin into a pale operated catfish whereupon it will darken, and the injection of adrenaline into a dark one which will then quickly blanch. These observations lead to the conclusion that the eyes of the catfish are not concerned with the discharge of intermedin.

Further evidence as to the control of intermedin in catfishes can be obtained from individuals whose central nervous organs are intact. When both eyes are removed from a normal, pale catfish and it is allowed to remain in the light, it soon assumes a coal-black tint irrespective of its background (Table I, 9, 10). Such a fish will apparently retain this maximum dark hue for an indefinite period (Van Heusen, 1917; Bray, 1918; Pearson, 1930; Parker, 1934; Abramowitz, 1936; Odiorne, 1937; Wykes, 1938; Osborn, 1938*b*). If a coal-black fish, rendered so by having been blinded, is transferred to darkness, it will cease to be coal-black, but will maintain a very dark tint (Table I, 12).[1] If a pale fish is completely enucleated and put at once into the dark, it will remain pale (Table I, 11). Both these fishes, however, when brought into the light soon become coal-black. I have shown elsewhere (Parker, 1940*b*) that the condition of the melanophores of such fishes in darkness is that of rest and that this stage of rest may be assumed anywhere between one extreme or the other in the whole range of color change. This stage of melanophore inactivity in darkness shows that the photoreceptors in the skin of such fishes are also in quiescence, for all such *Ameiurus* when brought into the light darken to full coal-black. In darkness, then, the photoreceptors in the skin of the catfish must be unstimulated, but when light falls upon them they are activated and thus induce the discharge of intermedin whereby the fish assumes a coal-black tint. That this darkening is due to a blood-borne agent is seen in the fact that when this test is carried out on a fish with a blanched, caudal band, the band darkens at the same time that the fish does.

[1] This statement is not wholly in agreement with the declarations of other workers. I have discussed these differences elsewhere (Parker, 1940*a*) and I shall refer the reader to this discussion for my reasons for the conclusion that blinded, coal-black catfishes in darkness are of very dark tints though not coal-black.

These observations lead to the conclusion that when light falls upon the integumentary photoreceptors of *Ameiurus* nervous impulses are set up for the discharge of intermedin and the consequent darkening of the fish (Abramowitz, 1936). They also show that these photoreceptors are fully quiescent in darkness. I therefore conclude in general that the discharge of intermedin is not the result of impulses from the eyes, but of impulses from the integumentary photoreceptors and that these receptors are stimulated to this effect when light falls upon them but not when they are in darkness.

From this standpoint the one chromatic activity of the photoreceptors in the catfish skin is to induce the discharge of intermedin. That this general control is a reflex operation there is every reason to assume. That it is not a nervous, spinal-cord reflex is clear, for no changes in color can be induced in a spinal catfish by the illumination of the skin on its flanks. In this my observations entirely agree with those of Wykes (1938). However, I do not agree with this worker in assuming that the reflex under consideration is a brain reflex in the way implied by her. Wykes assumes a type of reflex based upon an interpretation of the expanded state of melanophores in denervated pelvic fins whereby the color cells therein located are supposed to be in a condition of dispersed pigment in consequence of their liberation by the denervating cut from some central nervous activity. But this interpretation has already been shown to be untenable (Parker, 1934, 1940*a*). It is true that the reflex here discussed is, as Wykes remarks, a reflex that involves the brain, but the activating impulses are not reflected from that organ over nervous paths to the melanophores as she believes. As already shown, they impinge in the brain on the pituitary gland there to induce the discharge of intermedin. In this type of reflex only the afferent arm is nervous (from the integumentary photoreceptor to the pituitary gland), the efferent arm being humoral (from the pituitary gland by way of the blood and the lymph to the melanophore). I have recently called attention to this somewhat unusual type of reflex, part nervous and part humoral, and I have designated it in consequence of the different conditions of its two arms as a neurohumoral reflex (Parker, 1940*c*; 1940*d*). In conclusion it may be said that the discharge of intermedin by the pituitary gland in *Ameiurus* according to this general view is not excited through the eyes, but through the photoreceptors in the skin and by these photoreceptors only when they are illuminated, not when they are in darkness. Under such circumstances they are inactive.

If the skin photoreceptors in *Ameiurus* are limited in their chromatic function to exciting a discharge of intermedin from the pituitary

gland as a means of darkening the fish, what part do the eyes play in these color changes? It has already been shown that the eyes are not concerned with the discharge of intermedin, for a pale catfish with a transected brain but with eyes and pituitary gland intact will remain pale on a black, illuminated background. Do the eyes control the autonomic nerve-fibers by which the melanophores are made to disperse or to concentrate their pigment?

This question has been in large part answered by the work of previous investigators. The presence of dispersing fibers in the catfish, notwithstanding the doubtful expressions voiced by Osborn (1938*b*) and by Wykes (1938), appears to be well established on the basis of the revival of caudal bands (Parker, 1934; 1940*a*) and of the regeneration of caudal pigmentary nerves (Abramowitz, 1935). Dispersing fibers have been taken for granted in two of the most recent papers on this subject (Chin, 1939; Chang, Hsieh and Lu, 1939). Are these fibers activated from the photoreceptors in the skin, from the eyes or from both sets of receptors? This question cannot be approached from the standpoint of the fish with transected brain, for the cut in such an animal severs not only the nervous tracts from the skin to the pituitary gland, but also those from the eyes to the melanophores. It is best dealt with in hypophysectomized catfishes. Hypophysectomized catfishes lose the intermedin from their blood only slowly. According to Veil (1937) they blanch in about three days. Osborn (1938*b*) identified intermedin in the blood of catfishes by means of tests on pale fishes 70 hours after hypophysectomy. By testing the blood of hypophysectomized fishes on pale individuals or on catfishes of intermediate tint, I have found evidences of intermedin at three days after hypophysectomy but never after five days. When catfishes completely free from intermedin were needed for experimental purposes they were held as a rule for a full week after hypophysectomy before special tests were made upon them. As already stated, a hypophysectomized catfish thus seasoned is limited in its color responses. It is incapable of becoming fully dark (Parker, 1934; Osborn, 1938*a*, 1938*b*; Wykes, 1938; Enami, 1939) or fully pale (Veil, 1938; Enami, 1939; Parker, 1940*a*). In normal *Ameiurus* the fully dispersed macromelanophores, as already noted, have a diameter of about 145 microns and the fully concentrated cells one of about 40 microns. After hypophysectomy the diameter of these cells at maximum dispersion is about 70 microns according to Osborn (1938*b*) or about 100 microns according to my measurements (Parker, 1940*a*) and their minimum diameters some 50 microns. Thus by hypophysectomy the catfish has lost about half its range in color change. The half range that remains to the fish after the loss of its

pituitary gland must be attributed to its other chromatic activators which are in the main the nerves for the dispersion and the concentration of its pigment. The dispersing nerves, supposed by some workers (Parker, 1934; Wykes, 1938) to be the chief agents in darkening the fish, have been shown to be of secondary importance as compared with intermedin (Veil, 1937; Osborn, 1938*a*, 1938*b*; Parker, 1940*a*). The concentrating nerves have long been recognized as the main blanching agents. These general features of the catfish melanophore system must be kept in mind in the following discussion which will be concerned chiefly with the steps necessary for the activation of the two sets of nerves for the color responses.

If a catfish previously hypophysectomized is completely enucleated the system controlling its melanophore responses will be found to be reduced to the photoreceptors of the skin and their nerves, and the two sets of dispersing and concentrating melanophore nerves. These parts together constitute all that is necessary for a complete melanophore reflex. Are these parts so organized that such a reflex is possible?

Catfishes prepared in this way and allowed to recover fully from the operations live well for some weeks. They are very satisfactory material for color tests. Their general conditions are recorded in Table I, 13 to 16. When a hypophysectomized catfish is enucleated even though it is in the beginning as moderately pale as such a fish can be, it is likely to darken considerably as a result of the operation. This is probably due to handling (Parker, 1940*a*). After such treatment these fishes are as a rule about intermediate in tint (Osborn, 1938*b*; Wykes, 1938) though some of them remain paler. In the light they retain their particular tints apparently indefinitely and this retention is irrespective of the background on which they rest (Table I, 13, 14). If hypophysectomized catfishes as near pale and as near intermediate in tint as they can be made to assume by background adjustment are quickly and carefully enucleated and at once put in darkness, they retain their original tints without change so far as can be judged (Table I, 15, 16). Moreover, when they are brought into the light they do not seem to alter in color. All these observations point to hypophysectomized, eyeless catfishes as chromatically inert and open to almost no reflex color change whatever. Their melanophores are in what I have regarded as stages of rest (Parker, 1940*b*). That these color cells have not suffered any serious incapacity by the operations to which the catfish may have been subjected can be shown in their quick and characteristic responses of pigment concentration to injections of adrenaline and of dispersion to pituitary extract (Osborn, 1938*a*). What is lacking apparently in these inert fishes is the reflex connections between the afferent photoreceptor

fibers from the skin and the efferent dispersing and concentrating fibers to the melanophores. In the catfish the nerve connections left after the removal of the pituitary gland and the eyes are not organized for color reflexes and I therefore conclude that the autonomic dispersing and concentrating nerve-fibers of the melanophore system in *Ameiurus* are not open to activation by the photoreceptor fibers from the skin of the fish. These two sets of melanophore fibers, as has long been known, are activated through the eyes and if the results just detailed are correct these receptors are the only ones concerned with the activation of these two kinds of nerve-fibers.

Discussion

The melanophore system in *Ameiurus,* as represented by its macromelanophores, appears to be organized upon the following general plan. The chief receptors concerned with the activation of the melanophores are the eyes and the photoreceptors of the skin. The photoreceptors are stimulated not in darkness but only when light falls upon the skin and are not influenced by an experimental environment, black or white. They excite the discharge of intermedin from the pituitary gland and the consequent darkening of the fish. They are not concerned with the activation of the chromatic nerve-fibers, dispersing or concentrating. The eyes are receptors through which a background, white or black, makes itself felt in the system of color cells. Through the eyes are excited the concentrating autonomic nerve-fibers and the dispersing ones by which the pigment in the melanophores is gathered in the center of the color cell or scattered throughout its processes, blanching or darkening the fish. The eyes, therefore, are the receptors concerned with the discharge of adrenaline and of acetylcholine from the chromatic nerve terminals. The eyes are not so related to the discharge of intermedin, and yet it would probably be false to deny to them any influence over the pituitary gland. When a catfish becomes pale through the entrance into its eyes of light reflected from a white surface, its skin is also under reasonably bright illumination. The effect of this skin illumination would be to induce the discharge of intermedin whereby the fish would be darkened. There is, however, not the least evidence to show that in a brightly illuminated, white environment any darkening agent is present in a catfish. On the contrary, the fish under these circumstances is fully and completely pale as though under the undivided control of adrenaline. It is conceivable, of course, that there is under these conditions a neurohumoral competition between intermedin and adrenaline like that which has already been pointed out in the dogfish *Mustelus* (Parker, 1937),

but evidence to this effect, unlike that seen in the dogfish, is entirely lacking. It is much more probable that in illuminated white surroundings the stimulation of the eye excites only the concentrating nerve fibers and at the same time overcomes the discharge of intermedin due to the illumination of the skin photoreceptors by some action on the pituitary gland. From this standpoint the eye in a luminous white environment not only excites the discharge of adrenaline but probably inhibits that of intermedin. In this respect the eye very likely plays a negative but important part in the control of intermedin. This idea of chromatic inhibition has already been suggested by Zoond and Eyre (1934) and by Sand (1935).

This, in brief, is a rough outline of the melanophore system in *Ameiurus*. That it is correct in its chief features seems probable from the evidence presented. That it is in any sense complete is far from likely. Why should hypophysectomy limit color responses and how is this limitation accomplished at both the dark and the pale ends of the color range? Is excitement pallor (Abramowitz, 1936) a reflex and if so what are the receptors and the nerve paths? The same questions may be asked concerning darkening due to handling. What is the nature of the so-called inherent reactions of melanophores, a subject recently emphasized by several active workers (Abramowitz, 1936; Osborn, 1938*b*)? These and a score of other questions show how restricted our knowledge is not only of the melanophore system in *Ameiurus* but of the chromatic system in general. They invite further research.

Summary

1. The melanophore system in the catfish *Ameiurus* composed of epidermal micromelanophores and of subdermal macromelanophores is activated not through direct stimulation by light, by humors from the eye ball, nor by the activity of the pineal organ, but by nerve impulses received from the eyes and from the photoreceptors in the skin.

2. The skin photoreceptors are stimulated by light but not by darkness and are exclusively concerned with the discharge of intermedin from the pituitary gland whereby the catfish darkens (neuro-humoral reflex).

3. The eyes are differently stimulated by light from a black environment and by light from a white one. In the former they give rise to impulses that pass over the dispersing autonomic nerve-fibers, induce a discharge of acetylcholine, the dispersion of pigment, and the darkening of the fish. In the latter they originate impulses that pass over the concentrating autonomic fibers, induce a discharge of adrenaline, the con-

centration of pigment, and the blanching of the fish. Impulses induced through the eye by an illuminated, white environment probably also inhibit the discharge of intermedin from the pituitary gland.

4. A technique is described for the transection of the catfish brain at the level of the cerebellum whereby the eyes and their appended centers may be separated from the whole integumentary photoreceptor system and its centers.

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THE EFFECT OF GROWTH AND NUTRITION ON MITOCHONDRIA IN LIVER CELLS OF FUNDULUS HETEROCLITUS

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Introduction

In a recent study on *Triturus pyrrhogaster* (McCurdy, 1939) I found that starvation and re-feeding tend to produce definite changes in the mitochondrial picture of the liver cells. In the course of starvation, the elongated mitochondria which predominate in well-fed animals gradually give way to granular mitochondria, and these latter finally become predominant. When the procedure is reversed and starved animals are re-fed, the mitochondria change in the opposite direction, from granular to elongated. These mitochondrial changes, however, though they clearly depend upon digestive activity and degree of starvation in general, are more closely related to the amounts of fat and glycogen in the liver itself. It can be stated as a rule that in the liver cells of *Triturus* increase in the fat and glycogen content is accompanied by an increase in the proportion of elongated mitochondria, and decrease in the fat and glycogen content is accompanied by an increase in the proportion of granular mitochondria. There is thus an important relationship between the nutrition of cells and the form of their mitochondria.

The present study is concerned with two questions: (1) Does a similar relationship between mitochondrial form and the amount of storage products exist in a different species? (2) Does growth affect the morphology of the mitochondria?

Material and Methods

Fundulus heteroclitus was selected for this study because it was readily obtainable at Woods Hole during the spawning season and adults and larvae could easily be kept alive in the laboratory.

For the starvation experiment, adults were kept in two aquaria supplied with constantly running sea water. One set were fed regularly every day with clams cut into small pieces, four individuals for one

week and four others for two weeks, and then killed twenty-four hours after their last meal. The second set were denied food, four individuals for two weeks and four others for three, before killing. All animals were killed by decapitation, the body opened immediately and small pieces of liver excised and fixed.

For the growth experiment, larvae were obtained by stripping adult males and females and fertilizing the eggs. At frequent intervals the hatching larvae were collected and removed to separate bowls labelled with the time of collection. After the first twenty-four hours they were fed every day with bits of clam, finely macerated. Each day, from the time of hatching through the twenty-fourth day, a batch of larvae were fixed whole.

The fixatives employed were Regaud for mitochondria and fat and Lison's mixture (dioxan-picric acid-acetic acid-formalin) for glycogen. The stains were: for mitochondria, Heidenhain's iron-haematoxylin; for fat, using frozen sections, Sudan III; and for glycogen, Best's carmine. The methods were the same as those used and described in detail in the paper referred to previously.

Observations

A. Starved and Fed Adults

In all the well-fed adults elongated mitochondria are predominant, chiefly long filaments, some branched or racquet-shaped, though a few large granules are present. Fat and glycogen are abundant.

In all the starved adults granular mitochondria are predominant, though there are a good many beaded forms, and a few rods. Fat and glycogen are totally absent.

B. Growing Larvae

Mitochondria.—In larvae just hatched (average length 4 mm.) granular mitochondria predominate, though a few thick rods and drop-like forms are present. With larvae of increasing age there is a gradual increase in the number of elongated mitochondria until at twelve days (average larval length 6.5 mm.) these forms predominate. By twenty to twenty-four days (average larval length 7 mm.) the elongated mitochondria are filamentous and resemble the mitochondria of well-fed adults. Beaded mitochondria seem to be transitional forms, as in *Triturus,* since they do not exist in the newly-hatched larvae but begin to appear later and then disappear as the smooth elongated forms come into prominence.

Fat.—There is no fat in the liver cells until the third day after hatching, when a small amount appears in the cells adjacent to the blood capillaries. Previous to this stage the fat is limited to the yolk and the blood stream. Fat increases in the liver cells gradually from the third day on and by the twelfth day is abundant. The yolk persists until about the seventh day, decreasing gradually.

Glycogen.—No glycogen is present in the liver at hatching. It appears in small amounts after a day. Like the fat it gradually increases in amount and is quite abundant by the twelfth day.

Conclusions

It appears from the foregoing observations that in the adult *Fundulus* mitochondrial form is correlated with the presence or absence of storage products in exactly the same way as in adult *Triturus pyrrhogaster.*

Larvae just hatched have granular mitochondria in the liver cells as do starved adults. As they grow, elongated mitochondria appear and finally become predominant, as in the well-fed adult. Since, moreover, newly-hatched larvae have no fat or glycogen in the liver, but as they grow older fat and glycogen appear and increase in amount for some time, it can be stated that the course of change in regard to fat, glycogen and mitochondrial form during normal growth in *Fundulus* is the same as in the case of starved adults restored to normal by re-feeding.

The rule which applies to mitochondrial form in *Triturus pyrrhogaster* holds good for both adults and larvae of *Fundulus heteroclitus.* The form of the mitochondria stands in a definite relationship to the amount of storage products, and thus serves as an index of the nutritional state of the animal.

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GROWTH STUDIES ON CILIATES

VI. Diagnosis, Sterilization and Growth Characteristics of Perispira Ovum

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The growth characteristics of a number of species of ciliates have been reported recently in the literature. Most of these papers deal with saprozoic forms (Phelps, 1935, 1936, etc.) or with bacteria-feeders (Johnson, 1933; Kidder and Stuart, 1939*b*). The report which is to follow deals with observations of a quantitative nature made on the holotrichous ciliate, *Perispira ovum,* and represents the first attempt at an anlaysis of the growth characteristics of an obligate carnivore in bacteria-free medium, although Brown (1940) has published a brief account of some of the growth characteristics of *Leucophrys patula,* a facultative carnivore. Due to the fact that *Perispira* is not a common genus and its previous descriptions have been contradictory (Stein, 1859; Levander, 1894; Kahl, 1926), a rather complete description of its organization will be given. This description will serve to establish the identity of our experimental organism and should eliminate confusion in future investigations, regarding its specific designation. We agree with the views expressed by Taylor and Furgason (1938) and Furgason (1940) that the space devoted to a purely morphological description is warranted as a means of standardization of experimental material.

Material and Methods

During the summer of 1939 a number of specimens of *Perispira ovum* were found in a sample taken from a fresh water stream on Gifford St. in the town of Falmouth, Massachusetts. This sample contained many *Euglena* along with numerous other species of protozoa. The *Perispira* were isolated and maintained in mass culture, along with their associated bacteria and *Euglena gracilis,* for a period of two months before any attempt was made at sterilization. During this period care had to be taken to make frequent sub-cultures, as heavy bacterial growth proved to be detrimental to the *Perispira.*

[1] Aided by a grant from the Manufacturers' Research Fund for Bacteriology and Protozoölogy at Brown University.

Description of Perispira ovum

The ciliate is a member of the family Spathidiidae. It is evenly oval in shape, its size varying with the state of its nutrition. Well-fed organisms measure 65 μ–120 μ in length $\times$ 50 μ–110 μ in width, while starved ciliates are much smaller (30 μ–60 μ $\times$ 20 μ–45 μ). The most characteristic structure of the body is a spiral ridge, originating at the dorsal anterior portion of the body. From the point of origin the ridge bends sharply to the right and then spirals posteriorly toward the left, ending on the left side of the body near the posterior end (Fig. 1).

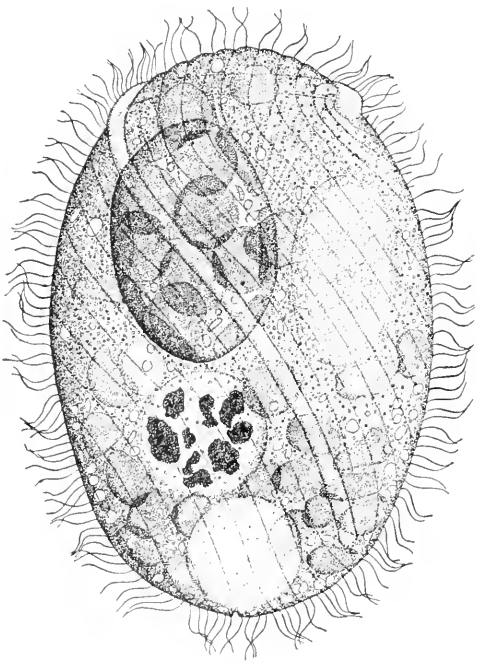

FIG. 1. Living ciliate, left side $\times$ 1000. The slender refractile trichites may be seen extending from the anterior end into the endoplasm. To the right of the drawing is the macronucleus; toward the posterior end are the contractile and excretory vacuoles, the excretory pore and the scattered chloroplasts and paramylum bodies. A single *Euglena* has recently been ingested.

Thus the ridge describes only one complete turn about the body, whereas Kahl (1926) figures an extra half turn. This ridge is composed of structureless, homogeneous protoplasm throughout most of its length, but in the anterior third it bears a narrow groove. This groove is slightly expanded very near its beginning into what appears to be a

small pore (Fig. 2). The pore and the groove represent the mouth, which is open only during the few seconds of food ingestion. The mouth can be seen in the non-feeding organism only after appropriate preparation (opal blue or nigrosin). Extending from the mouth and running into the endoplasm are a number of refractile and delicate trichites (Fig. 1). In most living specimens a few of these trichites may be seen lying loose in the endoplasm, but the majority are arranged as are those in a typical *Spathidium*. We have been unable to demonstrate them with any of the standard techniques. Levander (1894)

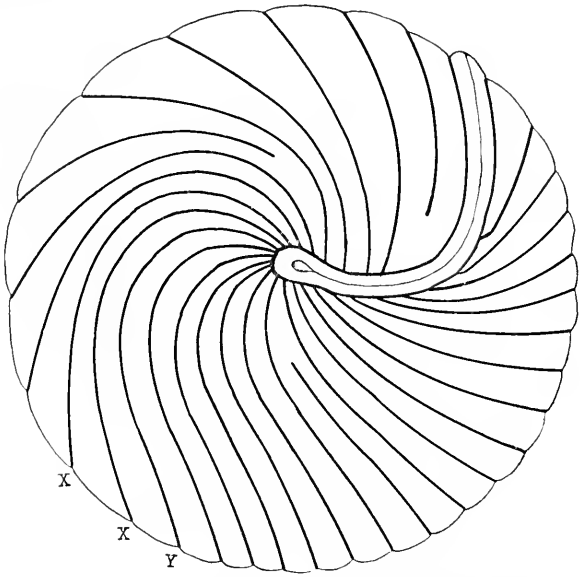

FIG. 2. Diagrammatic representation of the anterior pole, showing the ridge and the origins of the ciliary lines. The mouth is indicated in the ridge. *x*, *x* represent the ciliary lines which bear the paired, short bristles. *Y* represents the ciliary line which bears the long, unpaired bristles.

saw these trichites and included them in his figure but interpreted them as forming a basket. Kahl (1926) figures the trichites but refers to them as "Trichocysten" and describes them as being present in the protoplasm of the ridge. We have been unable to verify this last observation and there is no evidence from a study of the activity of a feeding animal that it possesses any trichocysts which function as do those in *Spathidium*.

The disposition of the cilia may be observed clearly in small organisms which are devoid of food. Relief staining methods with opal blue or nigrosin on medium-sized or small ciliates give excellent and

striking results and permit accurate analysis of the relative size and distribution of the motor organelles (Fig. 3, *A* and *B*).

On either side of the spiral ridge there is a row of closely set cilia. These rows join at the anterior end of the ridge but remain separate at its posterior end. This arrangement makes, in effect, a continuous row of cilia starting at the posterior end of the ridge, encircling the anterior end, and returning on the opposite side of the ridge. Contrary to the statement of Kahl (1926), we have found these cilia to be identical in size but much more closely set than the rest of the peripheral

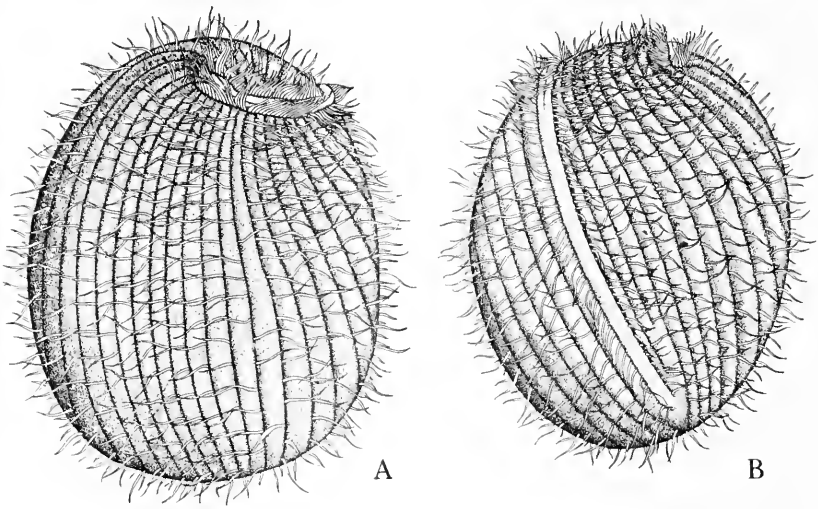

FIG. 3. Drawings of opal blue preparations of medium-sized organisms to show the size and arrangement of the cilia and the disposition of the ciliary rows. Organisms slightly flattened in preparation. × 700. *A*. Right side. Note the three rows, bearing bristles, toward the left of the drawing. *B*. Left side, showing the ridge.

cilia. It seems probable that these closely set cilia were mistaken by Kahl for the trichocysts which he describes as being present in the ridge (Fig. 3, *A*).

The general peripheral ciliary rows originate from the edges of the ridge in the region of the anterior pole. These rows spiral around the body from right to left, paralleling the ridge. They end irregularly in the region of the posterior pole. There are 26 complete rows of fairly widely placed cilia, with from one to three interpolated rows. The general arrangement of these rows may best be understood by referring to Fig. 2. Three of these peripheral rows deserve special attention, since they are made up of diverse types of structures. Two of the rows,

originating from the anterior tip of the ridge, bear short, paired bristles (Fig. 2, *x, x;* Fig. 3, *A* and *B*). These bristles are distributed along the first quarter of the length of the row and appear to originate from basal bodies directly in the row. They may be observed in living organisms and are clearly demonstrated after relief staining. They appear to vibrate rapidly but through extremely short arcs. They correspond to the "Chemoreceptoren" described by Gelei (1933) in *Trachelophyllum,* except that he maintains that their origin is between the ciliary rows. We cannot subscribe, at present, to Gelei's theory as to their function. There is no evidence that *Perispira* receives chemical stimulation through these bristles. Just to the right of the rows which bear the short bristles is a single row of long, stiff bristles (Fig. 2, *y;* Fig. 3, *A* and *B*). These are unpaired and occupy most, but not all, of the length of the row, which is completed posteriorly by normal flexible cilia. These long bristles correspond to the "Tastborsten" of Gelei (1933), but do not appear to function as tactile receptors, as he supposed they did in *Trachelophyllum.* Contact with prey in the region of these long bristles evokes no apparent response in *Perispira.* Another region where touch stimuli are received will be described shortly when the mechanism of feeding is considered.

The endoplasm of *Perispira* is coarsely granular and contains many different types of inclusions. Well-fed organisms are bright green due to the tightly packed chloroplasts of the ingested *Euglena.* The protoplasm of the *Euglena* is digested first, releasing the chloroplasts and the paramylum bodies. Next the chloroplasts are broken down and the unassimilated material deposited in a vacuole, as a reddish-brown mass, for defecation. Animals in the early stages of starvation contain quantities of paramylum bodies, which gradually disappear, leaving the *Perispira* relatively clear. These paramylum bodies appear to function as reserve food, enabling the ciliate to continue living long after all the *Euglena* have been ingested.

A single contractile vacuole is located at the posterior end of the body. It empties its contents to the outside through a permanent pore, which is always visible at the extreme posterior pole (Fig. 1).

The macronucleus is quite large and coarsely granular. It is variable in shape, being ovoid to elongate or even horseshoe-shaped. In starved animals it regularly breaks up into two spheres, as described by Levander (1894). After staining with haematoxylin, two types of granules are demonstrated, the fine chromatin granules and larger spheres of deeply-staining substances lying in vacuoles. Their appearance corresponds to that of the spheres found in such forms as *Paramecium*

(Wenrich, 1926) and *Diophrys* (Summers, 1935). After the Feulgen reaction, however, only the chromatin granules are stained and the vacuoles which hold the larger spheres appear hollow, giving the macronucleus an areolar appearance.

There is a single micronucleus in *Perispira ovum* which is very low

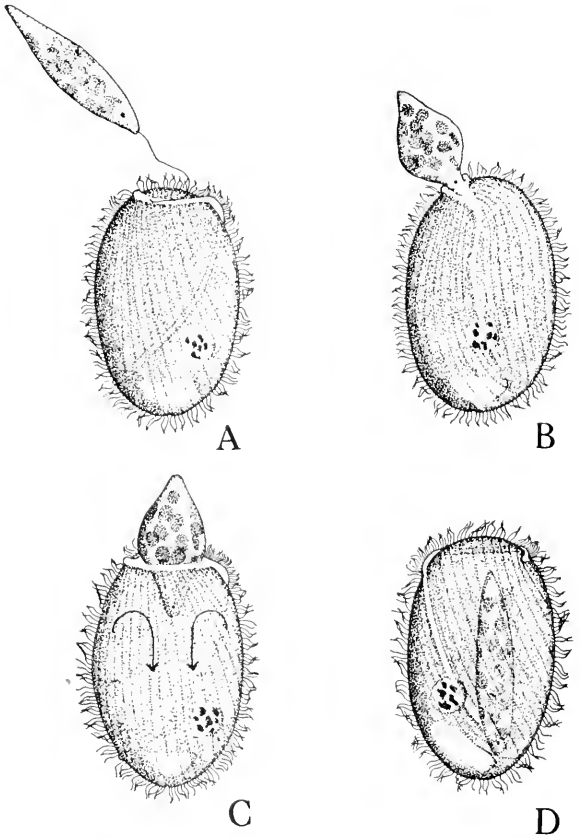

FIG. 4. Starved *Perispira ovum*, showing successive stages during the ingestion of a *Euglena*. *A*. Flagellum is caught. *B*. Mouth beginning to open. *C*. Arrows indicate the direction of the cyclosis which carries the *Euglena* in. *D*. Mouth closed immediately after ingestion.

in chromaticity. It regularly lies very close to the macronucleus and may easily be overlooked. Kahl (1926) states that he was never able to identify it with certainty.

We have made extensive observations on the mechanism of feeding. Food taking is best observed by placing a drop of a culture containing starved *Perispira* on a slide and adding to this drop a number of active

Euglena. Within a few seconds almost all of the ciliates will have started feeding and the whole process can be studied under an oil immersion lens.

The ciliates swim with a rotating motion which is uninterrupted by contact with other ciliates or with rounded *Euglena*. When the flagellum of an active *Euglena* comes in contact with any part of the ridge, however, the motion of the *Perispira* changes and it stops its rotation. If the contact has been made near the anterior end of the ridge (Fig. 4, *A*), the flagellum is rapidly drawn in, dragging the flagellate up to the mouth. The ridge immediately opens along the seam (Fig. 4, *B*) and the *Euglena* is drawn into the body of the *Perispira* (Fig. 4, *C* and *D*). The mouth then closes and the whole process may be repeated many times in the course of a few minutes. The force which draws the prey into the mouth is manifested by the increased intensity of the cyclosis of the protoplasm of the ciliate, which creates a current directed inward from the mouth opening (Fig. 4, *C*). Ingested *Euglena* are not surrounded by a distinct vacuole, but appear to lie free in the endoplasm of the ciliate. Killing of the prey is very slow and it is not uncommon to see four or five ingested flagellates contracting and expanding within the body of the *Perispira*.

Although we placed *Perispira* in fluid containing numerous species of protozoa, we have never observed it ingesting any types except euglenoid flagellates. It may be that its specialization has gone so far that its prey must possess certain structural characteristics in order to evoke the feeding response.

Sterilization and Establishment in Bacteria-free Culture

Advantage was taken of the fact that well-fed *Perispira* are positively geotropic. One to two milliliters of a heavy suspension was placed in the top of a special tube filled with sterile water (pH 7.0). The tube was constructed with alternating shelves in order to prevent any clumps of débris from falling to the bottom. After the majority of the ciliates had migrated around the shelves to the bottom of the tube they were drawn off into a sterile, cotton-plugged centrifuge tube. The ciliates were then centrifuged slowly until concentrated, the supernatant fluid withdrawn with a sterile serological pipette and more sterile fluid added. This washing process was repeated ten times, after which the ciliates were placed in the top of a 50 ml. burette full of sterile water. After this second migration had been completed and a large number of the ciliates withdrawn from the bottom of the burette into a sterile cotton-plugged tube, they were placed in a Syracuse watch glass

enclosed in cellophane (Kidder, Lilly and Claff, 1940). Single ciliates were then withdrawn by means of sterile capillary pipettes and placed into other similar dishes, from which they were then removed to tubes containing either water or 0.5 per cent Yeast Harris.

The above method yielded several sterile ciliates. The various steps described were necessary, inasmuch as this ciliate possesses rather uneven contours and is not easily washed free of its adhering bacteria. All cultures finally labeled as sterile were so designated only after the various sterility tests had been used in accordance with our previously described methods (Kidder and Stuart, 1939*a*).

The washed ciliates were placed into: (1) water (pH 7.0) or (2) 0.5 per cent filtered Yeast Harris. To both of these fluids sterile *Euglena* had previously been added. The method of sterilization of our strain of *Euglena* has been reported elsewhere (Kidder, 1940). Growth of the ciliates was rapid in some tubes but extremely slow in others. It soon became evident that the tubes in which rapid multiplication occurred were bacterially contaminated, while the others were sterile. We considered the possibility that the bacteria might be furnishing some substance needed for normal growth. Accordingly a suspension of *Phytomonas* (the contaminating bacterium from one of the growing cultures) from an agar slant was made in water (pH 7.0) and autoclaved for 20 minutes at 120° C.; 0.5 ml. of this suspension was added to a tube containing sterile ciliates in water with *Euglena*. A Seitz filtrate of a 24 hr. culture of *Phytomonas* in 0.5 per cent Yeast Harris was also prepared and 0.5 ml. added to another tube containing sterile ciliates in water with *Euglena*. In both tubes the ciliates immediately began to multiply and proved to be sterile.

Loop transplants were made from the first of these cultures into: (1) autoclaved *Aerobacter* in casein peptone (Peptone Roche) and (2) autoclaved *Phytomonas* in Yeast Harris to both of which *Euglena* had been added. Growth was good in both media, but in all cases appeared slightly better without Yeast Harris. Transplants from these cultures into Difco Tryptone and *Euglena*, without autoclaved bacteria, proved successful and the cultures have been carried in 1.0 per cent Tryptone since then.

After nearly a year of continued culture in Tryptone and *Euglena*, we feel sure that the function of the autoclaved bacteria and of the bacterial filtrate in our initial establishment experiments was simply to reduce the speed of the growth of the *Euglena* so that the single *Perispira* which were inoculated were not "overgrown." Later serial stock transplants were always made with many *Perispira* and the rapidly growing *Euglena* were quickly reduced in number.

Technique of Experimental Culture

All experimental cultures were grown in the special culture flasks described elsewhere (Kidder, 1941). These flasks are provided with a port plugged with a vaccine tip, which allows one to draw off samples for counting with little danger of contaminating the cultures. The volume of all cultures was 100 ml. Incubation was at 28° ± 1° C. The cultures were grown in a box provided with a large water filter behind which a constant source of light was provided by means of a 50-watt electric bulb.

The fluid to be used in any set of experiments was autoclaved, allowed to cool and inoculated with *Euglena gracilis* and placed in the light box for from 24 to 48 hours. Just before the *Perispira* were inoculated the concentration of *Euglena* was determined by drawing out a sample and making cell counts with a Sedgwick-Rafter counting chamber and a Whipple micrometer. From a culture of *Perispira* two to three weeks old, the average size of the individuals having been determined previously, inoculations were made through the side arm of the flasks. The initial inoculum was determined for each flask by immediately withdrawing a sample and counting the number of *Perispira* in a unit volume. These counts were made with the aid of a dissecting binocular microscope, using the same system as has been reported for *Tetrahymena* (Kidder, 1941). Thereafter samples were taken at regular intervals and the concentrations of *Euglena* and *Perispira* determined. The average individual volume of the *Perispira* was calculated from measurements of 50 cells. Before measuring, the cells were fixed in the fumes of osmic acid. The volume was calculated by considering the individual cell as a prolate spheroid and applying the formula $V = 4/3\ \pi\ ab^2$, where $2a$ = the major axis (length) and $2b$ = the minor axis (width).

Growth

Normal Population Curve

The growth of a population of *Perispira*, when they are inoculated into a flask of 1 per cent Difco Tryptone containing a concentration of *Euglena gracilis* of approximately 10,000 per ml., follows a reproduceable and characteristic course. The shape of the curve depends on a number of conditions, one of which is the age of the inoculum. If *Perispira* reproducing at their maximum rate (logarithmic growth phase) are used as the inoculum, they continue to reproduce at this rate in the experimental flask. When the logarithms of the population densities are plotted against time the result is a straight line. During this

TABLE I

Average data from five experiments. Medium, 1 per cent Difco Tryptone; age of inoculum of *Perispira* 15 days; *Euglena* grown in the medium 34 hours before the *Perispira* were inoculated.

	Time in Hours										
	0	18	30	42	60	90	114	139	162	186	204
Perispira per ml.	100	93	145	235	554	4250	22920	57800	78100	79160	77700
Euglena per ml.	14640	70820	159600	282800	393400	289800	48240	2096	—	—	—
Indiv. Vol. in $\mu 3$	21490	110750	147000	174400	206400	237400	126400	54750	36750	32440	31250

TABLE II

Medium—1 per cent Difco Tryptone; age of inoculum of *Perispira*—21 days; *Euglena* grown in the medium 48 hours before the *Perispira* were inoculated.

	Time in Hours												
	0	23.5	49.5	73	92.5	115.5	145	168	192.5	216.5	403	571	1003
Perispira per ml.	90	75	490	2000	10000	36500	85500	93500	112000	85000	62000	11750	350
Euglena per ml.	9500	53000	130000	255000	241000	11500	—	—	—	—	—	—	—

logarithmic growth phase the generation time (calculated from the formula $g = \frac{t \log 2}{\log b - \log a}$ where t = the time in hours during which the population has been increasing, a = the number of cells per unit volume at the beginning and b = the number of cells at the end of the time, t) is 10.5 ± .3 hours. When ciliates taken from the stationary phase (10 to 20 days old) are inoculated there follows a period of approximately

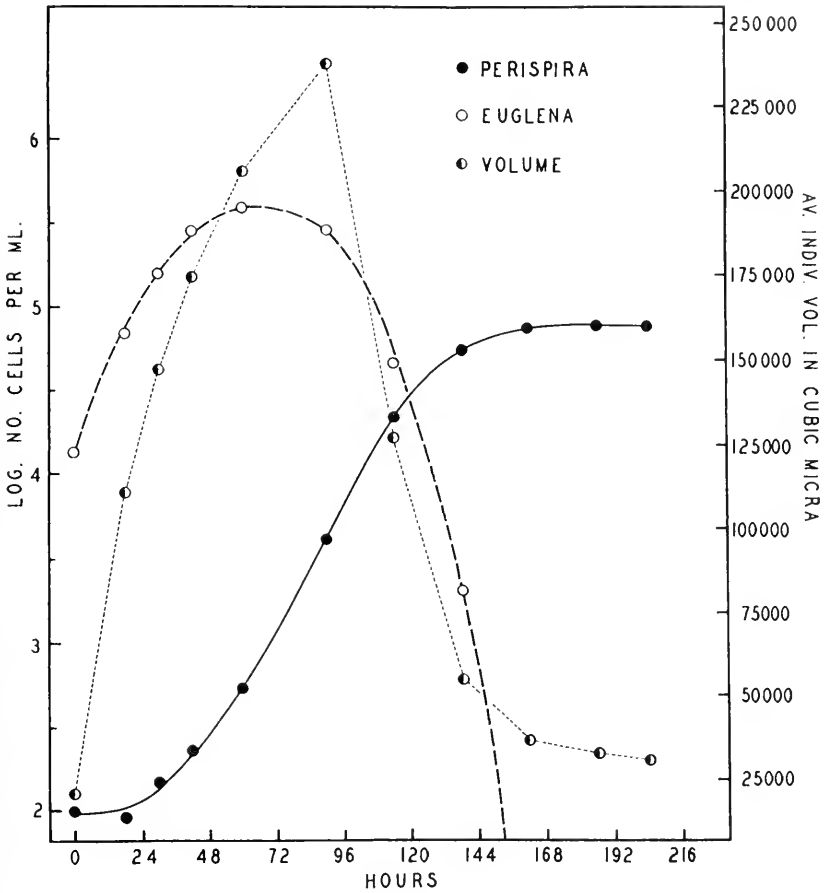

FIG. 5. Graphic representation of data presented in Table I.

24 hours during which there is no increase in the population. Usually some of the inoculated ciliates die during this lag phase. After a considerable number of *Euglena* have been eaten, reproduction follows and the culture goes into the logarithmic phase. The slope of the curve (and therefore the generation time) is identical with that for populations started with logarithmic ciliates (Table I; Fig. 5).

During the early phases of a culture the *Euglena* show a marked increase in concentration but the *Euglena* curve turns over rapidly as the *Perispira* increase. The *Euglena* curve represents the resultant of their own multiplication and their destruction by the *Perispira*. At about the 120th hour most of the trophic flagellates have been ingested (Fig. 5). These curves correspond, in general, to Fig. 6 in Brown's (1940) paper.

After most of the available flagellates have been eaten the *Perispira* continue to reproduce for some time at the expense of their individual size. Eventually the concentration reaches a relatively constant level at about 100,000 cells per ml. From then on the population declines slowly and there are still many small, trophic forms, capable of starting healthy cultures, even after two months. Table II gives the results of

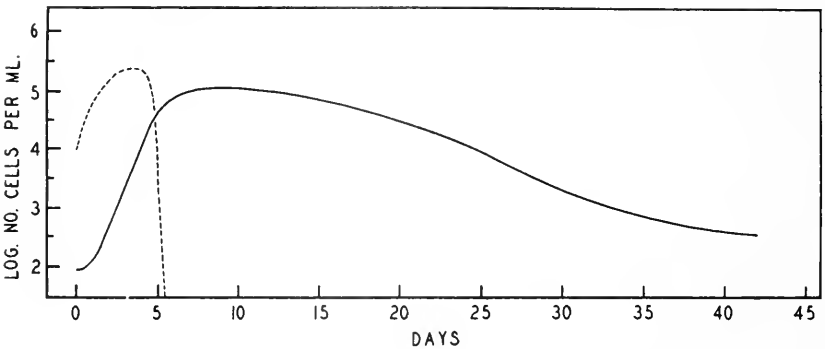

FIG. 6. Graphic representation of data presented in Table II.

an experiment carried for 42 days and these results are shown graphically in Fig. 6. Separate experiments, wherein large, medium-sized and small ciliates were selected for single cell inoculations, indicate that there is a relationship between the size of these starved cells and their ability to resume feeding and reproduction. This correlates with the deaths which occur upon mass inoculations into the experimental flasks. We believe that the variability in size during the late phases of a culture is due to purely fortuitous circumstances, however, and that the larger forms are those which were able to procure the last of the *Euglena*. Further investigations upon this point are needed.

Size Changes in Relation to Age of Culture

Ciliates taken from cultures three weeks old are very small and transparent. As was stated above, when these organisms are used as the inoculum a lag occurs before reproduction starts. It is during the

lag phase that the size of the individuals is increasing most rapidly. Feeding starts as soon as the *Perispira* and *Euglena* are brought together. The ciliates do not have to reach their maximum size, however, before they undergo reproduction. The individual size steadily increases during the logarithmic phase until the time when the food organisms begin to decrease in concentration. After this peak the size diminishes rapidly. The most rapid decrease in cell size corresponds to the upper limits of the logarithmic phase and the phase of negative growth acceleration. During the stationary phase the cell size is still decreasing, but very slowly (Fig. 5). This last and gradual decrease seems to be the result of the utilization of stored food materials while the previous rapid decrease was brought about largely by partition of protoplasm through rapid division.

The size changes hold for all types of media investigated so far and for the two species of euglenoid flagellates employed (*Euglena gracilis* and *Astasia klebsii*) as food organisms. Differences in speed of increase and maximum size attained were noted but these differences were slight.

Effect of Media, Light and Species of Food Organism

Having established the fact that the growth characteristics of *Perispira* in Difco Tryptone and *Euglena gracilis* were quite regular and reproduceable, experiments were set up to determine what effects would be produced by varying the standard combination. Flasks were prepared as before except that 1 per cent Difco Proteose-peptone or 0.5 per cent Yeast Harris was substituted for the Tryptone. In other flasks sterile *Astasia klebsii* was substituted for *Euglena;* still other flasks were prepared with colorless *Euglena* (grown in the absence of light) in Tryptone. All the flasks were inoculated with *Perispira,* the first three types were incubated in the light box while the last was incubated in a constant temperature box (27° C.) in the dark.

The differences observed between the various types of experiments may be summarized briefly. In the colorless *Euglena* there were a few more deaths among the individuals in the initial inoculum than in the controls. The generation times in the logarithmic phase were identical, however, and the maximum concentration and longevity of the cultures were not significantly different. In Proteose-peptone the general shape of the curve was similar to that of the control, but the generation time was 12.2 hours as compared to 10.6 hours for the control. The Proteose-peptone cultures declined more rapidly than did the controls. When the *Perispira* fed on *Astasia* the generation time was greater than

in the controls (12.5 hours), the maximum concentration was much lower (13,000 as compared with 93,000 per ml.) and the decline was very rapid. Moreover the *Astasia* were never completely removed from the culture, as shown by the fact that after the *Perispira* had become reduced to 40 per ml. (17 days) the *Astasia* had multiplied so that the concentration was again appreciable. These declining ciliates refused again to feed on the *Astasia*. In Yeast Harris the ciliates inoculated had nearly all died in 24 hours and in 48 hours all were dead.

General Observations

There are a number of comments to be made which do not have a place in the experiments described above.

When *Perispira* was first isolated and was growing in association with bacteria many monsters invariably appeared in the heavy cultures. These monsters were sometimes two to three times the size of the largest normally shaped individuals and were quite irregular in outline. They appeared to be the result of a failure to divide, on the part of certain individuals, while the power to ingest food was retained. After the establishment of our cultures in bacteria-free media only occasionally were monsters observed. When these organisms were encountered they were not used either in the counts or in the measurements. Attempts to start cultures from these monsters always failed.

Although there is no evidence that *Perispira* forms cysts, conjugates or undergoes endomixis, a peculiar type of reaction is characteristic of organisms in the later stages of a culture. About the seventh day after a culture is started a small percentage of the individuals become associated in pairs, superficially resembling conjugants. Upon closer examination, however, no protoplasmic fusion or even contact can be detected. Instead the long cilia of the anterior end of one individual appear to be stuck to the anterior portions of the ridge of the other, thereby holding the two organisms together. This association is rather loose and the two separate soon after they are placed on a slide. We do not know whether or not this reaction has any significance in the life history of the ciliate, but we are inclined to think that it may represent simply an increased stickiness of the ridge-ectoplasm during the initial period of food stortage.

We have evidence that indicates that the establishment of the normal reproductive rate of a freshly inoculated culture of *Perispira* is influenced by the time during which the food organisms had been growing in the medium before the inoculation of the ciliates. This is a conditioning effect and is being investigated for a future report. It is also

true that the physiological condition of the food organism determines, to a large extent, the ability of the *Perispira* to feed. Recently we have had cultures in which the *Euglena* (grown through many transplants in 1 per cent Difco Tryptone) were smaller than normal and possessed a reduced number of chloroplasts. Although these *Euglena* were active, the *Perispira* ingested few of them and as a consequence remained relatively small and reproduced very slowly. When these ciliates were presented with normal flagellates, however, they began to eat rapidly. In order to keep our food organisms in good condition, therefore, it seems necessary to maintain stocks in an acetate-containing medium, such as Medium D described by Hall (1937).

Discussion

The establishment of *Perispira ovum* in bacteria-free culture along with *Euglena gracilis* as the food organism affords an opportunity for the study of a carnivore under controllable conditions. The fact that cultures remain alive for only a short time when in association with their natural bacterial flora, while sterile cultures last for months, emphasizes again the harmful effects of some types of bacteria. This situation was pointed out in a previous work (Kidder and Stuart, 1939*a*) on a bacteria-feeding species and seems to hold equally well for this carnivore.

An analysis of the reproductive rate and the rate of increase in cell size is interesting. The lag phase is not a period of inactivity. The ciliates are feeding rapidly and increasing in size at a higher rate than at any other time. This is also true of the majority of bacteria (Henrici, 1928), although the mechanism of size increase appears to be different (for a discussion of this point see Stephenson, 1939). A similar analysis of the size changes of *Leucophrys* would be interesting. No data on this point are given in Brown's (1940) paper. During the first part of the logarithmic phase the size measurements indicate that reproduction, while it certainly decreases cell size (by one half), does not keep pace with individual growth resulting from feeding. These ciliates may be said to eat faster than they divide. After the concentration of the food organism begins to decline there is no decrease in reproductive rate, so that, for the first time, reproduction overtakes and passes feeding rate. Experiments wherein food organisms would be continually added to the culture would be very interesting in this connection. Such experiments are planned.

Factors limiting the growth of carnivorous ciliates, grown as described above, have not been analyzed. Under the conditions of our

experiments perhaps the most important single factor is food, but there are many others. As compared to a culture of a purely saprozoic species these carnivores offer certain advantages and certain disadvantages for growth studies. One of the most complicated factors is an evaluation of the balance between the growing food organism and the carnivore. We will be nearer a solution of some of the problems when we formulate a medium which will not support multiplication of the food flagellates, but will maintain them in a trophic condition. So far none of the media used satisfies these requirements.

Summary

1. *Perispira ovum* is a member of the family Spathidiidae. It is carnivorous, feeding on euglenoid flagellates.

2. A morphological description is given in order to establish the identity of the experimental organism.

3. A description of the mechanism of feeding is given. *Perispira* possesses a spiral ridge to which the flagella of euglenoid flagellates adhere. The flagellum and then the body of the prey is drawn into the mouth of the ciliate where it eventually rounds up and is digested. The cytoplasm of the prey is digested first, then the chloroplasts and lastly the paramylum bodies.

4. *Perispira* was rendered bacteria-free and established in culture with sterile *Euglena gracilis* as food.

5. When the population of such a culture is followed, a typical growth curve results. If ciliates from an old culture are used as the inoculum there follows a period of lag, a logarithmic growth phase, a phase of negative growth acceleration, a stationary phase and a phase of slow decline.

6. Cell size increases during the lag phase and early logarithmic phase but decreases rapidly during the late logarithmic phase and phase of negative growth acceleration. Cell size is correlated with the presence of food and the rate of cell division.

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THE DEVELOPMENT OF A COLONIAL ORGANISM: SYMPLEGMA VIRIDE

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The compact, closely related group of polystyelid ascidians, including the botryllids, are all able to produce buds, either to form social aggregates or true colonies. In either case the manner of budding is fundamentally the same, namely, by the protrusion and development of localized regions of the atrial epithelium together with its overlying epidermis. At the same time, the nature and form of the colonies or groups are intimately related to the differences, with regard to this phenomenon, that do exist among the various genera. A comparative study of bud production and bud development within the polystyelids is illuminating, and of these ascidians *Symplegma viride* is one of the least studied and the most interesting. It is typically West Indian in distribution, including southern Florida and Bermuda; it is also common in the East Indies and neighbouring waters.

A colony, in part, and a mature zoöid are shown in Fig. 1, together with a representation of the relationship of the buds in a growing margin of a colony. In size, thickness, and colors, and in size of constituent zoöids, *Symplegma* and *Botryllus* are much alike. They differ in some important respects. The zoöids in *Symplegma* are not arranged in coördinated groups or systems, and each zoöid has its own independent atrial siphon. Moreover, unlike *Botryllus,* the constituent zoöids of a colony of *Symplegma* persist while the colony lives and are not brief transients. As in *Botryllus,* vascular ampullae crowd the colony margins and exhibit rhythmical contraction. In a large colony little evidence of budding can be seen. In small and growing colonies, buds at various stages of development may be numerous in some regions of the colony margin. These may be seen in Fig. 1, *B*.

Mature zoöids are considerably larger than those newly formed by budding. Colonies, in fact, consist mainly of three types: small colonies with numerous buds and relatively small sexually immature active zooids; larger colonies with larger zoöids as yet sexually immature, and little sign of buds; and large colonies, again without buds, consisting of relatively large sexually mature zoöids. Each zoöid is connected with the common vascular system of the colony by a ventral stolon.

Apart from numerous differences in form and detail, the mature zoöid of *Symplegma* is markedly different from that of *Botryllus* with regard to gonads and buds. In *Symplegma* buds are not present on mature zoöids, while the gonad contains a maximum of about eight

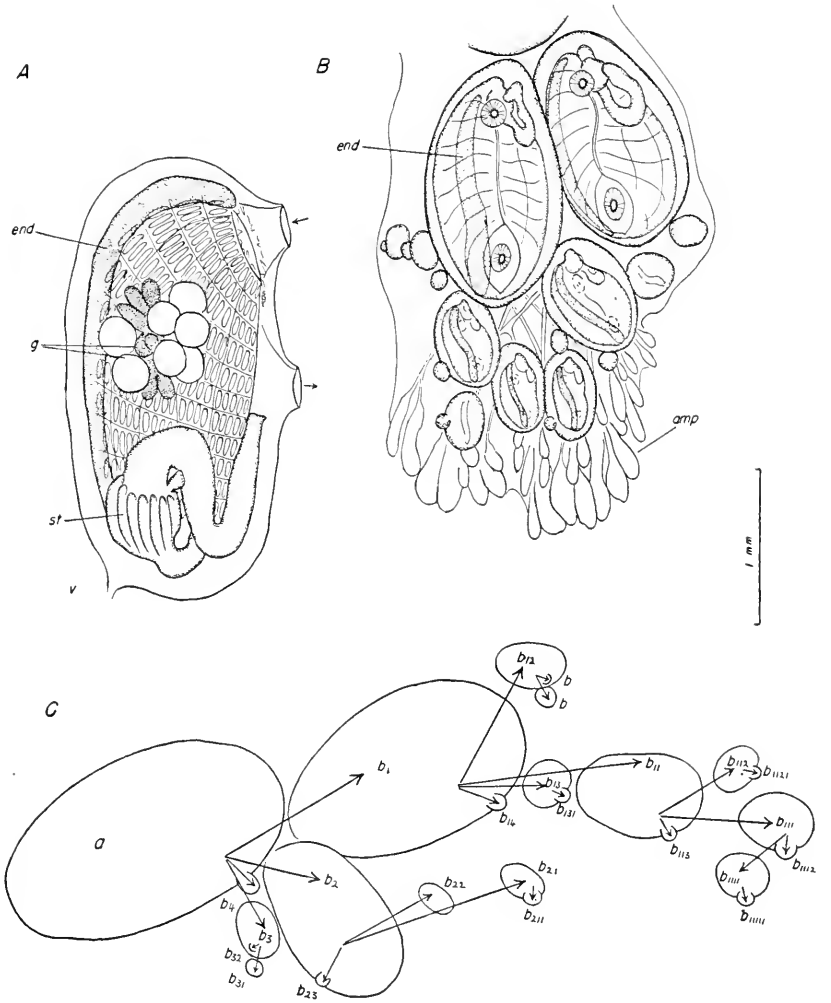

FIG. 1. Mature zoöid of a colony (*A*), and two camera lucida drawings of marginal growing zone of young colonies, at the same scale (*B* and *C*). The arrows in *C* indicate the origin, in sequence, of the buds produced in the course of development of each bud, all the buds shown having evolved directly or indirectly from the now active bud (*a*).

amp, vascular ampullae of colony; *end*, endostyle; *g*, gonad; *st*, stomach; *v*, ventral vessel connecting zoöid to colonial circulatory system.

large eggs as compared with the maximum of four characteristic of *Botryllus schlosseri.*

Development of the Egg

The fertilized egg develops in the atrial cavity of the parent and emerges as a swimming tadpole larva. The larva is typically botryllid in structure and activity, as has been emphasized by Caswell Grave (1932), although the degree of development of the permanent ascidian structure of the trunk region is relatively much less at this stage (cp. Berrill, 1935). The tadpole larva metamorphoses into a sessile zooid attached by eight ventral ampullae just as in *Botryllus.* It differs from the equivalent stage of the last form in that there are only four primitive gill slits (protostigmata) on each side of the pharynx. It also differs in that at this stage there is no sign of buds.

In any given generation of *Botryllus* zoöids there is no increase in number of rows of gill slits or number per row, once they have appeared in the developing zoöid. In *Symplegma,* on the other hand, the oozoöid grows continually in size, and correlated with this expansion (see Fig. 2) the primary gill slits subdivide into rows and in addition new subdividing stigmata successively appear in the ventral region of the pharyngeal sac. The general increase in the complexity of the branchial wall and the ampullary circulatory system external to the zoöid is shown in Fig. 2, *F.* At this stage two small buds have already appeared. These are shown at a larger scale in Fig. 2, *G.* The first of these has already lost its original attachment to the parent and has acquired its secondary connection by means of its ventral stolon with the ampullary circulatory system. The second and smaller bud is still attached by its wide stalk to the parental point of origin. In all of the several post-larval forms examined the relationships of the two buds at this stage were identical and there is no doubt that the buds all arise from a specific region of the body wall, namely on the right side near the base of the right ampullary vessel where it passes into the body wall. At this early stage therefore a rudimentary colony already exists.

The subsequent development of the colony can only be understood in the light of the development of the bud itself. It is already evident however, that compared with *Botryllus,* buds appear relatively belatedly, on one side of the body only, in series rather than as a single bud, and lose primary connection with the parent zoöid extremely early. The following section accordingly deals with bud development from a dual point of view, as a morphogenetic process and as a process of budding in relation to the developing colony.

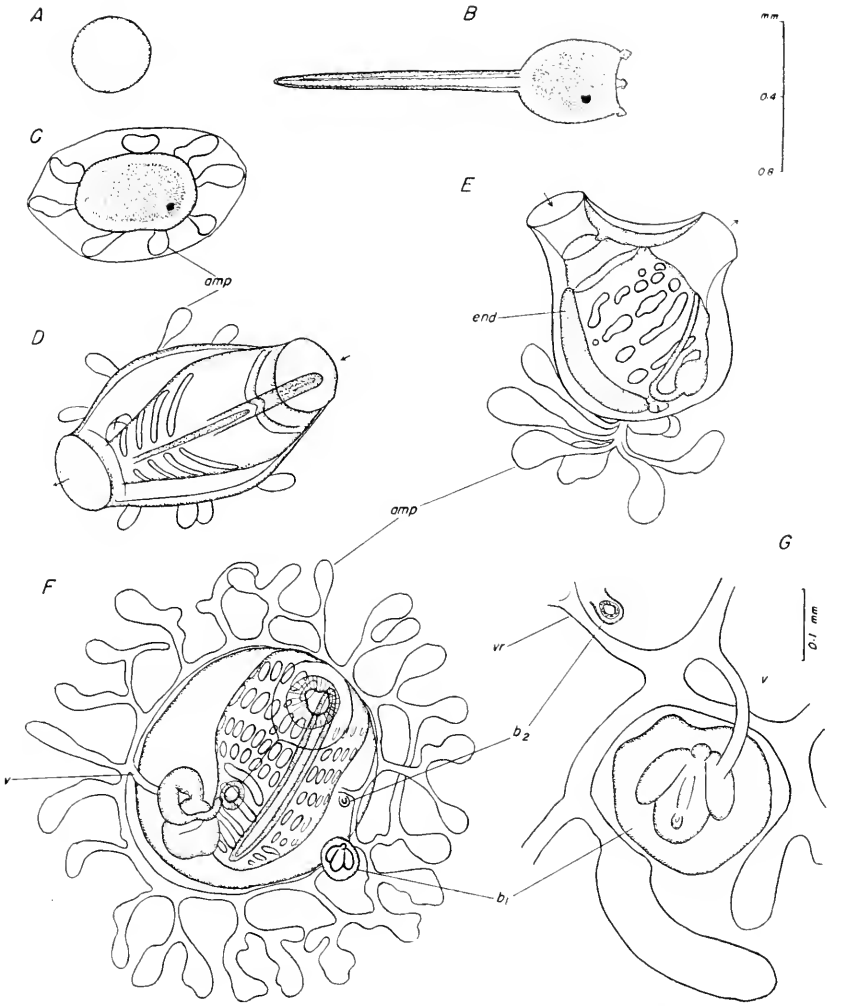

FIG. 2. Development from egg to first colonial organism. *A*, egg; *B*, swimming tadpole, showing three adhesive organs and single complex sense organ; *C*, metamorphosing stage with tail absorbed and eight vascular ampullae growing out as organs of attachment; *D*, first functional stage of ascidiozoöid shown in *C* with open siphons, and active gill splits in form of protostigmata; *E*, same individual as in *D*, after some growth, with protostigmata transforming into rows of definitive stigmata; *F*, later stage of same showing further subdivision of protostigmata, elaboration of ampullary system, and appearance of first two buds; *G*, the two buds and their relationship to the vascular ampullary system, at a higher magnification, the smaller still attached at its point of origin at the base of the right ampullary vessel, the larger having lost its original connection and acquired a secondary attachment through its own left ampullary vessel.

amp, ampulla; b_1, b_2, first and second buds; *end*, endostyle; *v*, left ampullary vessel; *vr*, right ampullary vessel.

Development of the Bud

Development of First-generation Buds

The development of the buds arising from the oözoöid (the zooid formed by the metamorphosed tadpole) can be described only partially. Three early stages of buds of the second series, and one later stage of buds of the first series, are available. These are shown in Fig. 3, and in general represent the two stages of buds shown in

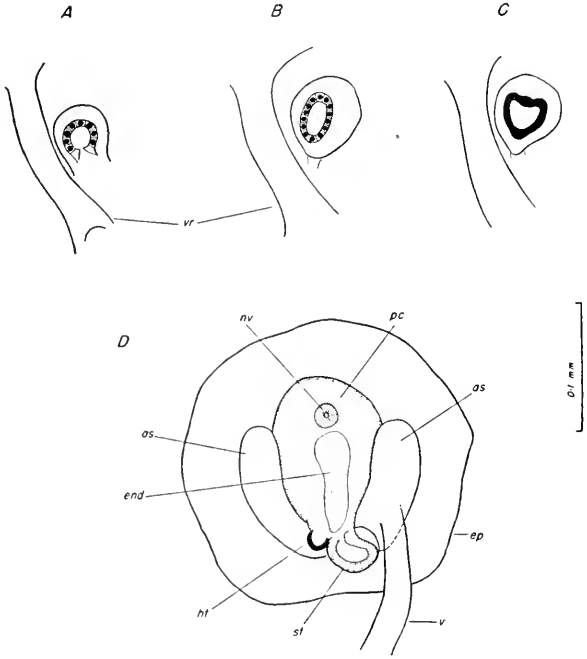

Fig. 3. Buds borne by primary individuals (oözoöid) of stage shown in Fig. 2, *F*.

A, B, C, three stages of second bud. *D,* first bud.

as, atrial sac; *end,* endostyle; *ep,* epidermis; *ht,* heart; *nv,* neural vesicle; *pc,* pharyngeal chamber; *st,* stomach; *v,* left ampullary vessel secondarily connecting bud with ampullary system of parent; *vr,* right ampullary vessel of parent near base of which all buds arise.

Fig. 2, *F* and *G*. The three smaller examples are shown in relation to the base of the right ampullary vessel, indicating the constancy in point of origin. In the smallest bud of the three the primary vesicle is not quite closed and the bud as a whole is united with the parental tissue by a wide stalk. The outer layer of the bud is continuous with the parental

epidermis and the inner layer with the atrial epithelium, just as in *Botryllus*. In the next largest bud the inner vesicle is a closed hollow sphere, while the outer layer is almost so, being attached to the parent by only a very narrow stalk. The stalk is still present in the third bud of the small series, but the inner vesicle is not only a little larger but already shows indication of subdivision into the essential organization units. The pair of folds that subdivide the chamber into the central pharyngeal and lateral atrial divisions can be seen just formed at the anterior margin, while a posterior thickening of the wall foreshadows the outgrowth of the intestinal region. In the later stage shown in Fig. 3, *D* these divisions are completed, and the central chamber has become further differentiated. The posterior thickening visible in Fig. 3, *C* has formed an intestinal outgrowth; endostylar grooves are forming on the ventral wall of the main sac, while an anterior and a right-posterior diverticulum or evagination represent the developing neural mass and heart respectively. The original stalk of attachment has disappeared and the bud has acquired a secondary connection, with the parental (now colonial) vascular ampullary system by means of an outgrowth from its ventral endostylar region.

Development of Buds in Older Colonies

The preceding section concerned mainly the origin and early development of the buds resulting in the conversion of the original individual developed from the fertilized egg into a real, though minute, colonial organism. Further details of bud development have been obtained only from the study of buds in larger and older colonies. The early stages of these are shown in Fig. 4. There is one significant difference between the following account and the one just given, quite apart from extension. The buds formed by the oözoöid arise from a fully differentiated and functional individual. The buds to be described arise from developing buds that are far from having attained the functionally differentiated state. In Fig. 4, therefore, each complete stage represents two bud generation stages and the relationship between them.

In Fig. 4, *A* the bud borne by the larger bud is at the stage of a disc in the process of becoming a hemisphere. In Fig. 4, *B* and *C* this process is continued until a hollow sphere, of atrial origin, is constricted off. A residual part of the original disc remains posteriorly in its original position.

In Fig. 4, *D* the epidermis enveloping the atrial vesicle is still widely connected with the parental epidermis. At the same time, in spite of

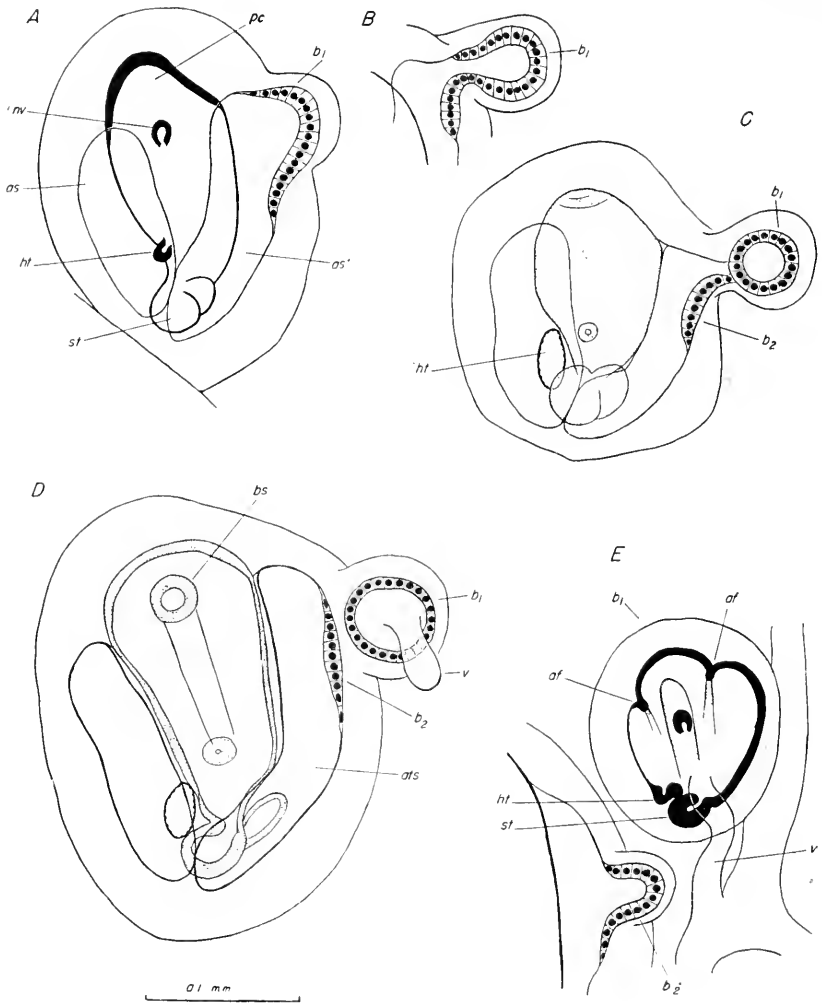

FIG. 4. Development of buds of advanced colony, as illustrated in Fig. 1, *B*. *A*, a bud at approximately same stage as in Fig. 3, *D*, bearing in turn a bud of the succeeding generation, arising as a convex thickening disc of the right anterior atrial wall.

B, *C*, later stages in formation of bud shown appearing in *A*, showing formation of closed vesicle and residual area of disc.

D, three stages, a development of the larger zoöid shown in *A*, its first bud now a closed vesicle with a developing ampullary vessel, and a residual disc representing the first stage in bud development.

E, more advanced stage, with residual disc forming second vesicle and further residual area; first bud has acquired attachment to colonial circulatory system by its ampullary vessel and is completely separated from its parental bud.

af, atrial folds; *as*, atrial sac; *ats*, atrial siphon; b_1, b_2, first and second buds arising from larger bud shown in *A*; *bs*, branchial siphon; *ht*, heart; *nv*, neural vesicle; *st*, stomach; *pc*, pharyngeal chamber; *v*, ampullary vessel.

the fact that the inner vesicle is still a simple hollow sphere, the epidermis opposite one side of it has grown out as a vascular ampulla. This, as may be seen in the next stage (Fig. 4, *E*) joins the vascular

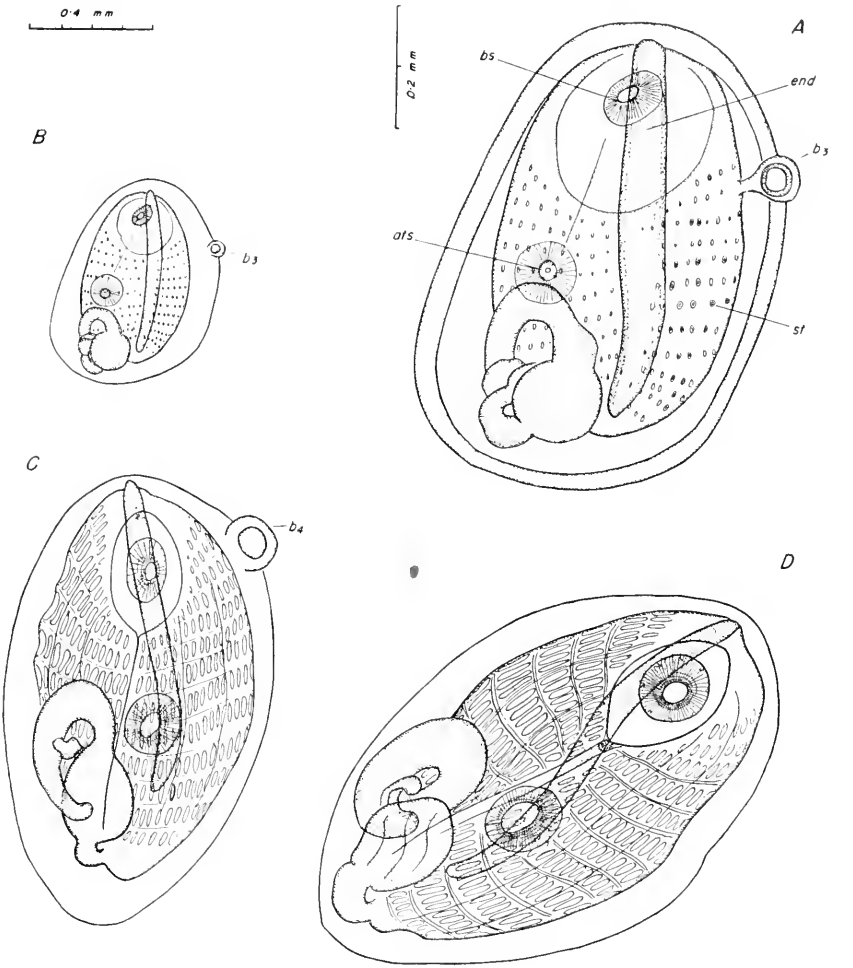

FIG. 5. Later development of bud up to fully active stage.

A, stage with rows of newly perforate stigmata, with third bud of series nearly separated. *B*, same stage shown at the reduced magnification of the succeeding stages. *C*, later stage giving rise to fourth and last bud. *D*, active stage complete except for absence of gonads, with no sign of further bud production.

circulatory system of the colony, and during the same period the bud is completely constricted off from the parent, epidermally as well as atrially. At this stage the main divisions of the organization are already

visible. The primary vesicle is being divided into the central and two lateral chambers by a pair of folds progressing antero-posteriorly. The diverticula or pouches representing the neural mass, heart, and intestine respectively have all appeared, and the endostylar limits are also visible. Later stages are shown in the parent buds illustrated in Fig. 4, *A*, *C*, and *D*. They are considerably larger but have not increased markedly in morphological differentiation. At the same time the process of budding exhibits some important features. The residual part of the disc giving rise to the buds described above becomes constricted off from the main part of the disc, as may be seen in Fig. 4, *B*, *C*, and *D*. This residual disc tissue grows somewhat in correlation with the growth of the parent bud and later transforms into a hemisphere, as in Fig. 4, *E*. This becomes a sphere and separates off to form a second independent bud just as in the first case, and again leaves behind a residual area of disc tissue in continuity with the atrial epithelium.

Continued development of the bud from the larger or parental bud shown in Fig. 4, *D* is to be seen in Fig. 5. Figure 5, *A* is drawn to one-half the scale of Fig. 4, and for spatial reasons the later stages are drawn to one-quarter of that scale. For purposes of comparison Fig. 4, *B* represents on the lesser scale the same stage shown in Fig. 4, *A*. At this stage much more morphological detail is apparent. The stomach and intestine are well differentiated, the siphons clearly marked, and the rows of numerous stigmata already perforate. A third bud, from the second residual disc area, is shown on the point of constricting off from the parent. In Fig. 5, *C*, at a stage where growth to the functional or active condition is almost reached, a fourth bud arising from the third residual disc area is to be seen constricting off. As far as can be determined, this represents the last of the bud series. In Fig. 5, *D* is shown the bud when it first attains the functional state, with open siphons and beating cilia in the gill slits. There is no sign of any further residual bud disc tissue. At this stage histodifferentiation is virtually completed, and the process of budding is to be associated with the developmental phase of the parent bud.

No sign of gonads has been detected in the newly functional zoöids. They develop during the subsequent active phase and mature in zoöids that are from two to three times in linear dimension the size they are when just active.

In Fig. 1 is shown in outline the zoöids in a marginal actively budding region of a young colony, showing the twenty-four buds arising directly or indirectly from zoöid *A*. The relationships are shown by the arrows.

Conclusions and Summary

As in *Botryllus,* the development of *Symplegma* is primarily the development of a colony and the development of an individual zoöid is modified accordingly.

Each developing zoöid or bud in the course of its development gives off a series of four buds from the anterior wall of the right atrial chamber. Individual zoöids when first active are less than one-half the final size and do not possess visible gonads. These appear later during the active or functional phase of growth.

As each bud disc develops into the vesicle stage, a residual part of the disc is unincorporated and remains to give rise to the next bud of the series, after a short period of growth. The production of buds thus comes to an end when growth of the parental organism as a whole is more or less complete.

The development of a bud, apart from the production of further buds, is an extremely direct process. A disc transforms into a hollow sphere. Two folds divide the sphere into a central (pharyngeal) and two lateral (atrial) chambers. Three pouches push out of the central division and give rise to heart, neural mass, and intestine. Later development is essentially an elaboration of these unit regions. Gill slits appear only when the pharyngeal wall has grown to a size permitting the simultaneous appearance, in miniature, of *all* the gill slits.

Buds, at a very early stage, lose their original connection with the parent organism and acquire a connection with the colonial circulatory system by means of a ventral stolonic vessel.

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OBSERVATIONS AND EXPERIMENTS ON THE PHYSIOLOGY OF MEDUSAE

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Since the classical work of Romanes (1885) and the experiments of Morse (1906, 1907), Murbach (1903, 1907, 1909), Yerkes (1902, 1904, 1906), and Yerkes and Ayer (1903) on *Gonionemus,* there have been very few studies of the behavior and other general aspects of the physiology of hydroid medusae. Gemmill (1919) gave an account of the flagellar currents of *Mericertum,* Harvey (1921) studied bioluminescence in several Puget Sound medusae, Weese and Townsend (1921) tested the reactions of *Aequorea* to pH, temperature, and salinity, and Milne (1938) and Edney (1939) have published some notes on the behavior of fresh-water medusae. During a stay at the Oceanographic Laboratory, Friday Harbor, Washington, in the summer of 1938, I was led by the great abundance of hydromedusae in those waters to make some observations upon several matters. My principal object was to study the course of food in the gastrovascular system, a matter on which, so far as I am aware, no exact knowledge was available. Some other points were also investigated.

As material, five common Puget Sound medusae were utilized: *Aequorea aequorea, Halistaura cellularia, Phialidium gregarium, Stomotoca atra,* and *Sarsia mirabilis.* These species can be obtained near the dock in front of the laboratory buildings in great abundance at practically any time. Some observations were also made on *Gonionemus vertens* and the stalked scyphozoan *Haliclystus sanjuanensis.* The animals were studied immediately after being brought in from the Sound or in many cases were observed directly from the dock.

Water Content

Recently (1938), I presented some data on the water content of *Aurelia,* and reviewed the available literature. As nearly all the data concern scyphozoan medusae, it seemed desirable to obtain some figures on hydroid medusae. Accordingly, freshly collected specimens of *Aequorea, Halistaura, Phialidium,* and *Sarsia* were given a quick rinse in fresh water, dried on paper toweling, and weighed to the third place

in previously weighed beakers. They were then dried in an electric oven kept at 100° C., and the drying was completed to constant weight in a desiccator over $CaCl_2$. *Aequorea* and *Halistaura* are large species and were determined singly. In the case of the small species *Sarsia* and *Phialidium,* a number of specimens were used for each determination, as shown in Table I. The smallest available specimens of *Aequorea* were also determined for comparison with fully grown animals and two

TABLE I

Water content of Puget Sound hydroid medusae, arranged in order of decreasing weight. Salinity of sea water, 3.09 per cent.

Large Aequorea				Halistaura			
No. animals	Wet weight	Dry weight	Percentage water	No. animals	Wet weight	Dry weight	Percentage water
1	34.303	1.182	96.56	1	20.422	0.709	96.53
1	31.594	1.092	96.55	1	20.389	0.709	96.53
1	27.976	0.960	96.57	1	19.091	0.655	96.57
1	24.787	0.843	96.60	1	14.402	0.497	96.55
1	20.963	0.726	96.54	1	13.265	0.457	96.56
Small Aequorea				Sarsia			
2	8.669	0.296	96.59	5	2.350	0.080	96.60
3	12.647	0.387	96.94	6	2.549	0.090	96.47
2	6.395	0.204	96.81	22	8.343	0.269	96.78
3	8.555	0.258	96.99	7	2.459	0.085	96.55
3	8.522	0.273	96.80	18	2.838	0.090	96.83
2	5.667	0.194	96.58				
3	7.101	0.241	96.61	Phialidium			
3	6.346	0.205	96.77				
3	6.136	0.211	96.57				
3	4.589	0.134	97.08	12	4.923	0.165	96.65
				13	5.137	0.147	97.14
				18	6.975	0.243	96.52
				20	6.313	0.207	96.71
				15	4.558	0.157	96.56
				18	4.104	0.136	96.69

or three were weighed together in each case. The data are presented in Table I.

These data show that the water content of Puget Sound medusae ranges from 96.5 to 97 per cent and is therefore similar to that previously found for other medusae (Hyman, 1938), although perhaps slightly higher. The salinity at Puget Sound was stated to be 3.09 per cent, hence slightly lower than that at Mt. Desert Island, Maine, where

Aurelia was found to have a water content of around 96.2 per cent. As previously pointed out (Hyman, 1938), the water content of medusae depends on the salinity of the sea water in which they are living, but unfortunately the salinity is often not stated in the literature. It is, of course, possible that hydroid medusae have a slightly higher water content than scyphomedusae but without concomitant data on the salinity of the sea water this cannot be decided.

The data in Table I suggest that young *Aequorea* have a slightly higher water content than grown ones. Statistical analysis shows that the difference in the water content of the small compared with the large *Aequorea* is significant, being 3.54 times its standard error.[1] It may therefore be concluded that the water content of medusae declines with increasing age (size) and this result is consistent with what is known of the water content of other animals. The data also indicate that the small species (*Phialidium, Sarsia*) have a slightly higher water content than the large ones (*Aequorea, Halistaura*) but the difference was found to be without sufficient statistical significance.

Dr. Earl Norris, of the chemical staff of the Oceanographic Laboratory, permits me to quote the following analysis of *Aequorea* made in his laboratory: water content, 96.45 per cent, salts (ash) 2.70 per cent, and organic matter 0.85 per cent. The salt content thus appears to be less than that of the surrounding sea water and indicates some degree of control by the animal of the passage of materials through its surface.

General Behavior

The behavior of these medusae was found to be correlated in many respects with their morphology. *Stomotoca* and *Sarsia* are Anthomedusae with a tall, narrow bell, and long pendent manubrium. *Aequorea, Halistaura,* and *Phialidium* are Leptomedusae, with a broad flat bell and a very short manubrium. The bell is relatively thin and flexible in *Phialidium* and *Halistaura,* so that it contorts with each pulsation, especially in the latter, but *Aequorea* has an unusual amount of jelly, rendering the bell stiff and inflexible, except at the margin.

Observed from the dock, all the species showed the same general behavior. All pulsate at an even rate for a period of time, then cease pulsations, float in any position for a while, then resume pulsations; and this alternation of activity and quiescence continues indefinitely. As is well known, the rate of pulsation is more rapid, the smaller the animal. The periods of pulsation and quiescence appeared to have no absolute

[1] The statistical calculation was kindly made for me by Mr. Daniel Lehrman, using Fisher's t-formula.

or relative time value [2] except in *Stomotoca,* which regularly pulsates 3–8 times in succession and then pauses for an interval equal to 1–4 pulsations. The degree of extension of the tentacles bears no relation to the bell activity, i.e., the tentacles might be contracted or fully extended in either pulsating or floating specimens. It has been reported for some medusae (i.e., *Gonionemus,* Yerkes and Ayer, 1903) that when they touch the surface, they turn over and float downward in an inverted position. No trace of any such behavior was seen in any of the five species listed. Touching the surface has absolutely no effect upon them.

Reactions to touch were tested by a blunt glass rod, mostly on medusae in the water reachable from the dock. The exumbrellar surface is in general highly insensitive and a light to moderate touch usually elicits no response. A tap or blow on the exumbrella results, in all the

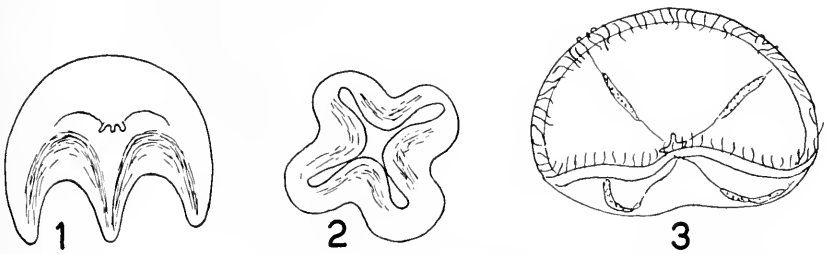

Fig. 1. Crumpling reaction of *Phialidium,* seen from the side.
Fig. 2. Crumpling reaction of *Phialidium,* seen from above.
Fig. 3. *Phialidium* giving the feeding reaction.

species except *Aequorea,* in a form of escape reaction which I call "crumpling." [3] The animal ceases pulsations, folds in the bell to the smallest possible compass (Figs. 1, 2), and sinks. The very stiff bell of *Aequorea* is incapable of such a reaction and, in fact, *Aequorea* gives no response even to severe blows which knock it for several inches, except that it may miss a pulsation. Bumping into objects often evokes the crumpling reaction.

Mechanical stimulation of the bell margin, if effective, evokes either an escape reaction or the feeding reaction. The escape response may consist of the crumpling behavior noted above or of bell pulsations. The sensitivity of the margin appeared to vary with species. In *Hali-*

[2] Romanes (1885, p. 147) has recorded the number of pulsations and the duration of the rest periods in seconds for three specimens of *Sarsia* and also found no correlation between the lengths of active and quiescent periods.

[3] What Romanes (1885, p. 123) called a "spasm" in *Staurophora,* "a sudden and violent contraction" causing the bell to assume a cuboidal form, is probably the same response as here termed "crumpling."

staura even a light touch often induced crumpling, whereas in the other species, a light touch was often ineffective. In *Phialidium* and *Sarsia,* marginal stimulation of the floating animal often resulted in the resumption of pulsations. *Stomotoca* and *Aequorea* were relatively insensitive to marginal stimulation. Strong marginal stimulation commonly results in crumpling in all forms except *Aequorea.* This animal, as already noted, is anatomically incapable of this type of escape reaction; to marginal stimulation, it either responds not at all or gives the feeding reaction, described below. Marginal stimulation may cause a cessation of swimming in a pulsating animal (*Phialidium*); this may be a form of feeding response. In the anthomedusan forms (*Sarsia, Stomotoca*), the manubrium, which hangs down below the level of the bell margin, appeared to be much more sensitive than the latter and would respond by crumpling or the feeding reaction to an intensity of touch which failed to evoke any response on the margin.

Response to light has been recorded for some medusae (*Gonionemus,* Morse, 1907; *Sarsia,* Romanes, 1885). In the forms here observed not the slightest response could be noted when they passed from a sunlit region into the shadow of the dock nor was any tendency ever seen to collect in either shaded or sunlit areas. The distribution of these medusae appeared to be determined wholly by the tidal currents and movements.

The water of Puget Sound near the laboratory is very cold, around 10° C. Any rise of more than a few degrees above this temperature diminishes the irritability of the medusae. At temperatures of 15–18° C., they tend to lie upon the bottom of the vessel in a flaccid, unresponsive condition, although pulsations continue. Weese and Townsend record that pulsations cease at 21–23° C.

Some medusae exhibit a food-catching ("fishing") behavior, swimming to the surface, then floating down, either right side up or inverted, with tentacles fully extended (*Gonionemus,* Yerkes and Ayer, 1903; *Limnocnida,* Edney, 1939). No food-catching behavior of any sort was witnessed in the Puget Sound medusae. They appear to depend wholly on chance contacts with suitable animals.

In the presence of food (giving off juices) *Gonionemus* shows excitement and makes random "searching" movements. The Puget Sound medusae, when placed in a vessel with a crushed bit of animal flesh, generally gave not the slightest sign of response even when quite close to the food. Occasionally, however, *Phialidium* when near the food would "hover" for a brief period. It seems probable that these medusae are capable of perceiving animal juice in the water.

The Feeding Reaction

To mechanical or chemical stimulation of the bell margin, all the species observed may give the feeding reaction. Mechanical stimulation consisted in touching the bell margin with a glass rod or forceps or moving the rod or forceps for a short distance along the margin. As already noted, such stimulation often evokes no response or may cause an escape reaction. Chemical stimulation consisted in touching a bit of animal flesh held in a forceps to the margin. As sources of food, fish, barnacles, *Mytilus,* crabs, etc., were used. The tentacles practically always grasp food and may grasp an inert object; the feeding reaction nearly always follows grasping of food by the tentacles, but does not invariably occur. If the feeding reaction fails to take place, the tentacles drop the food after a time.

The nature of the feeding reaction is correlated with the morphology of the animal. In the Leptomedusae (*Aequorea, Halistaura, Phialidium*) and also *Gonionemus* (Yerkes, 1902) with broad shallow bells, the reaction consists in the bending of the stimulated part of the margin to the manubrium (Fig. 3). The manubrium, which is very short in these forms, also moves to meet the inbent margin. The margin is held against the manubrium for a period during which the manubrium usually, but not invariably, grasps the food. When this has occurred, the margin returns to its normal position. If the manubrium fails to attach to the food, the margin will usually drop the food after a time. An *Aequorea* was observed in which the margin with food attached repeatedly bent to touch the manubrium until finally the manubrium grasped the food.

The following experiments were performed on *Phialidium,* which proved the most suitable form for studying the feeding reaction. If while one part of the margin is performing the feeding reaction, i.e., is being held against the manubrium, another part of the margin is stimulated, it too will give the reaction, providing the interval between the two stimulations is not too short. A third point of the margin may also be induced to respond; although stimulation of a third point may instead release another feeding reaction from the first region stimulated. The fact that the animals often fail to respond makes it difficult to study these reactions in detail or to investigate the question of fatigue.

The feeding reaction in the Anthomedusae (*Sarsia, Stomotoca*) differs from that of the Leptomedusae. These animals have a tall narrow bell and a long manubrium hanging below the level of the bell margin (Figs. 4, 5). The feeding reaction consists in the bending of the manubrium towards the stimulated part of the margin. The margin

does not show any movement. The manubrium was not seen to touch the margin but simply to make a nearly right-angled bend towards the stimulated region. In case of food held by the tentacles, the manubrial lip would touch and grasp the food.

In a leptomedusa which he called *Tiaropsis indicans* (stated by Mayer, 1910, to be probably *Eutimium socialis*), Romanes (1885) found that the tip of the manubrium would be precisely applied to any strongly stimulated spot of the bell. No such exact reactions were seen in any of the Puget Sound medusae.

In all the species observed, the lip of the manubrium (mouth frill) was found to be far more sensitive to chemical stimulation than any other part of the body. Food touched to the mouth frill was invariably

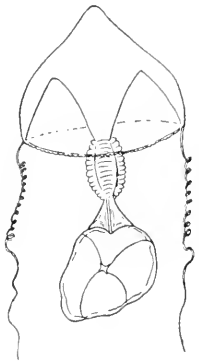

Fig. 4. *Stomotoca* with mouth frill attached to a *Phialidium*.

grasped. Probably the mouth frill in forms with a long manubrium is the chief agent in food capture.

Very few of the numerous medusae observed around the laboratory docks were seen to contain ingested food. It seems probable that the food requirements of these animals are low. *Sarsia* was never seen with ingested food. *Stomotoca* regularly attacks other medusae and was not infrequently seen with the expanded mouth attached to *Phialidium* (Fig. 4), sometimes to the larger forms. *Stomotoca* was also observed attempting to capture a crustacean larva, which escaped. *Phialidium* appeared to feed chiefly on small or minute plankters but might ingest larger crustacean larvae. *Aequorea* appeared to be a somewhat indiscriminate feeder and was observed with ingested crustaceans, *Nereis,* and peas and salmon refuse from adjacent canneries. Following a night run of a small *Nereis, Aequorea* and *Halistaura* would be seen on the next morning with ingested *Nereis*.

Course of Food in the Gastrovascular System

As already mentioned, the chief purpose of these observations was to study the function of the gastrovascular system. To my knowledge, no proof exists that food is actually carried along the canals of this system, although statements to this effect are standard in textbooks. To study the matter, bits of fish, barnacles, *Mytilus*, or crabs were dipped into a thick paste made of powdered carmine and sea water and then touched to the mouth frill of medusae. Such bits were invariably grasped and usually, although not always, swallowed in a short time. *Stomotoca* and *Sarsia* proved rather refractory subjects and would often fail to ingest the piece of flesh. The course of the ingested material is easily followed by means of the red carmine.

Within half an hour after ingestion red material is seen spreading along the radial canals and within two or three hours it occupies the entire gastrovascular system. The material appears to be driven along the canals chiefly by the bell contractions. At each contraction, the material moves back and forth or swirls about. Gemmill (1919) in *Melicertidium* (correct name, *Melicertum*) found definite flagellar currents, running peripherally along the roof, centrally along the floor of the radial canals. Examination of Puget Sound medusae under the microscope failed to show any definite currents in the gastrovascular canals, although the flagella could be seen in active motion.

Immediately the food reaches any region of the gastrovascular system it is subject to ingestion by the gastrodermis,[4] where it undergoes intracellular digestion. Surprisingly enough, however, the rôle of the gastrodermis in intracellular digestion varies greatly throughout the gastrovascular system. The chief sites of intracellular digestion are the gastrodermis of the manubrium and stomach (cavity at the upper end of the manubrium) and of the tentacular bulbs (basal swellings of the tentacles). These regions soon become stained deeply pink from ingested carmine particles (Figs. 5, 6, 7). Radial, circular, and tentacular canals take up some particles but play a minor rôle in comparison to manubrium, stomach, and tentacular bulbs. In forms such as the Leptomedusae which bear the gonads on the radial canals, those portions of the radial canals adjacent to gonad tissue were good sites of intracellular digestion; in non-gonadal regions the radial canals ingested very few carmine particles (Figs. 6, 7). In *Sarsia,* an expanded region near the manubrial tip is termed the stomach and is, in fact, the place where the ingested food is held, whereas in most medusae the stomach is an

[4] I have elsewhere introduced the term *gastrodermis* for the inner epithelium of coelenterates, usually called entoderm, in the belief that the terms ectoderm and entoderm had best be restricted to embryonic stages.

expanded region in the summit of the bell, where the radial canals originate, and the food is passed into this chamber although, if too large, part of it remains in the lumen of the manubrium. In *Sarsia* it was found that the entire manubrium has as good digestive powers as the so-called stomach region (Fig. 5) and this was also the case with the other medusae. In short, the gastrodermis not only of the stomach but of the whole manubrium has high powers of intracellular digestion, equalled only by the gastrodermis of the tentacular bulbs. Sections through the tentacular bulbs of medusae showed that the gastrodermis is here highly columnar and packed with food vacuoles.

It is well known that in some hydroid medusae the radial canals open to the exterior by a pore near the bell margin (Fig. 7). These pores have been presumed to be excretory. In *Aequorea,* which possesses such pores, strands of mucus with entangled carmine particles were seen exuding from these pores a couple of hours after carmine feeding. This observation indicates that the pores may serve for the ejection of indigestible material.

Carmine particles persist in the gastrodermis for at least two or three days after feeding. The fed medusae were not retained long enough to determine the time interval required for complete elimination of the fed carmine.

Gastric Juice of Aequorea

Following the example of Beutler (1924), gastric juice was collected from *Aequorea* by feeding the animals small bits of sponge soaked in animal juice. Since it was soon learned that the juice of *Mytilus,* barnacles, etc., contains enzymes, all such juice was thereafter boiled. Small bits of boiled bath sponge were soaked in such boiled juice and then touched to the mouth frill of *Aequorea,* which always grasped them. Ingestion usually followed, but not infrequently the piece of sponge would be soon ejected. The experiments were not as satisfactory as hoped, because even when retained, the piece of sponge was seldom taken thoroughly into the gastric cavity, partly because of the incompressibility of the sponge and partly because of the stiff thick jelly of *Aequorea.* However, in many cases the sponges were retained in the manubrium for a considerable period. The pieces of sponge were then withdrawn with a forceps and their contained fluid expressed into a beaker. Some simple tests for enzymes were made with this fluid on litmus milk, bits of boiled animal flesh, and starch solution. The presence of a protease capable of dissolving animal flesh and of a lipase were indicated but amylase appeared to be absent.

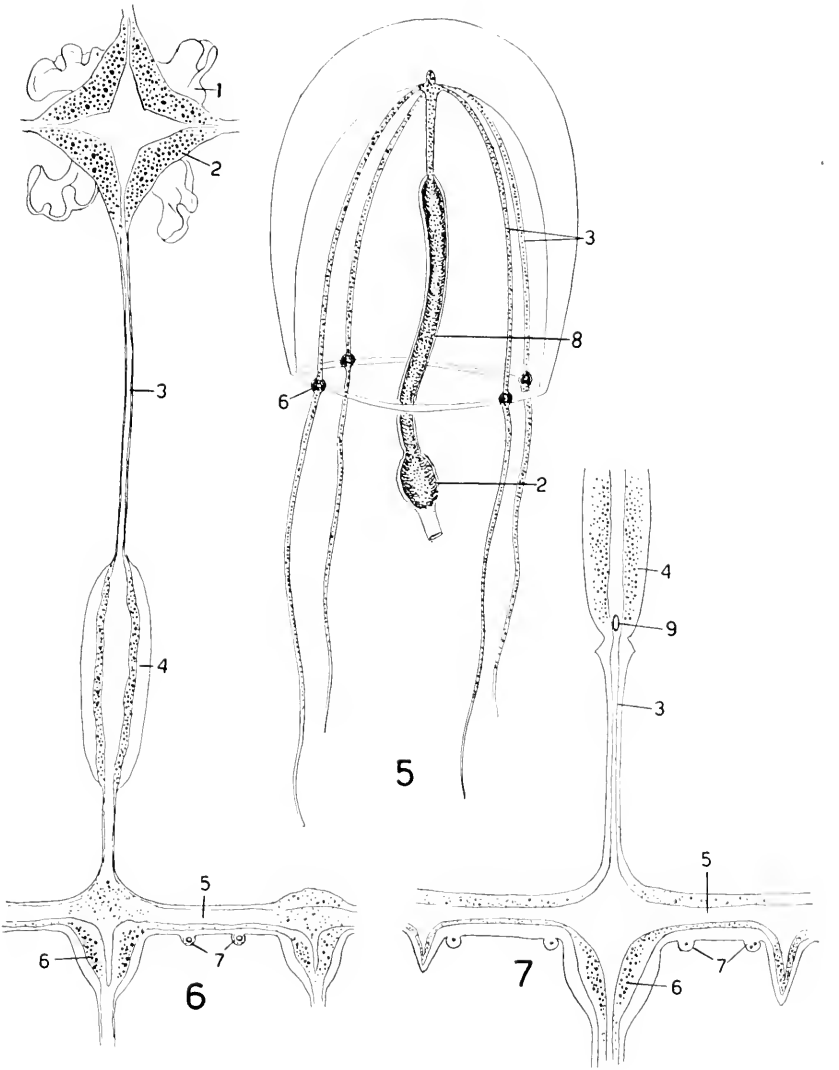

FIG. 5. *Sarsia*, following ingestion of carmine, showing distribution of carmine in the gastrodermis.

FIG. 6. *Phialidium*, stomach, one radial canal, and piece of margin, showing distribution of carmine in the gastrodermis, six hours after ingestion.

FIG. 7. *Aequorea*, piece of margin and adjacent part of one radial canal, showing distribution of carmine in the gastrodermis. Abbreviations: *1*, mouth frill, *2*, stomach, *3*, radial canal, *4*, gonadal region of radial canal, *5*, circular canal, *6*, tentacular bulb, *7*, statocysts, *8*, manubrium, *9*, so-called excretory pore.

A number of *Aequorea* containing an ingested prey, chiefly *Nereis,* were captured and the gastric fluid removed by a fine pipette. Such fluid was in all cases but one more acid than the sea water, which has in Puget Sound a pH of 8.0–8.2. Of 12 individuals, the gastric juice was 7.2 in 1 case, 7.4–7.6 in 9 cases, 7.8 in 2 cases, and 8.0 in the twelfth specimen. A piece of *Mytilus* placed in the combined juice from four animals, with a pH of 7.4–7.6, was much dissolved in three hours, while a control piece in sea water was unaffected. The possibility, of course, remains that the acidity of the gastric juice might have come from the disintegrating prey.

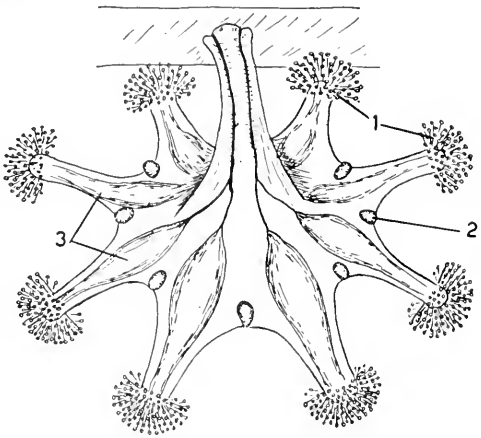

FIG. 8. *Haliclystus,* pendent from a seaweed, seen from the aboral surface, showing anchors and arms with tentacle clusters. *1,* tentacle cluster, *2,* anchor, *3,* gonads.

BEHAVIOR OF HALICLYSTUS

The stalked scyphozoan, *Haliclystus sanjuanensis,* one of the Stauromedusae, is common on seaweeds in a shallow bay of San Juan Island. During exceptionally low tides one can wade out and observe this interesting form in its natural surroundings.

Haliclystus (Fig. 8) has a trumpet-shaped body with the flaring oral end scalloped into eight arms, each terminating in a ball of small capitate tentacles. In the bottom of each scallop is an oval body, called the anchor, which, according to Schlater (1891), is provided with sensory cells and mucous glands. Seen in their natural habitat, the animals are fastened by the pedal disk to seaweeds and hang in a pendent attitude with the anchors erected. The medusa is relatively inactive. The chief activities seen in nature were: swinging on the stalk, flicking of the tentacles, sudden bending of an arm to the manubrium, or the folding

of all arms against the manubrium. When touched with a glass rod, the tentacle clusters might not react but often grasped the rod tenaciously and executed the feeding reaction. This consists of the quick bending of an arm so that the tentacle cluster touches the manubrium. It is very quickly given when food is touched to the tentacle cluster and arms other than the one touched may join in the reaction. The chief food in nature appeared to be the amphipod *Caprella*, common on the seaweeds where *Haliclystus* lives.

Brought into the laboratory, *Haliclystus* does not remain in good condition very long. Attempts to feed the animals with carmine-coated *Caprella* failed. The tentacles quickly grasped the food and bent with it to the manubrium which accepted it; but ingestion never ensued and the food was dropped after a time. Detached *Haliclystus* made strenuous efforts to reattach themselves, using the tentacle clusters for adhesion, but never succeeded even when placed on seaweeds. The tentacle clusters are extensively used in adhering to objects. No function could be discovered for the anchors. They were utterly unresponsive to mechanical or chemical stimulation, even severe pushing about, and although always credited with adhesive powers, were never seen to be employed by the animal for this purpose. The tentacle clusters appeared to be the most sensitive part of the animal. When touched they adhere and usually give the feeding reaction. Mechanical stimulation of the manubrium was ineffective; touching the subumbrellar surface usually evoked the feeding reaction in the adjacent arm. Strong mechanical disturbance of the animal may result in the infolding of all the arms, a response resembling the crumpling reaction of free medusae. Flicking of the tentacles or bending of the arms toward the manubrium were often witnessed without apparent cause; perhaps microscopic food is being caught at such times.

The Function of the Tentacular Bulbs of Hydromedusae

The tentacle bases in hydroid medusae are commonly enlarged, forming the tentacular bulbs (Figs. 6, 7). The function of these bodies appears to have been frequently misunderstood. Because in some Anthomedusae each bulb bears an ocellus, the entire bulb is often called an ocellus in books and laboratory manuals. The bearing of an ocellus is, however, only one function of the tentacular bulbs and in fact not their usual function.

My study of the tentacular bulbs of a number of medusae has shown that the epidermis of these bulbs contains much sensory epithelium and is also a depot for the manufacture of nematocysts (these functions were already known). The epidermis is crowded with cnidoblasts in process

of secreting nematocysts and there can be no doubt that the tentacular bulbs furnish nematocysts for the rest of the body. I have also discovered, as already noted in the discussion of intracellular digestion, that the gastrodermis of the tentacular bulbs plays an important rôle in this process. This gastrodermis is a tall columnar epithelium which after food ingestion becomes packed with food vacuoles. The intense colors often seen in the tentacular bulbs and frequently carefully described in taxonomic accounts represent food materials in the gastrodermis of the bulbs and hence are of no systematic value.

In *Gonionemus* and other trachymedusae, the tentacular bulbs are often very large and conspicuous and partially or wholly detached from the tentacle bases. The tentacular bulbs of *Gonionemus murbachii*, found in the Eel Pond at Woods Hole, contain a black pigment and this has led to the erroneous idea that these bulbs are ocelli or "eye-spots." Thus in the last edition of Drew's "Invertebrate Zoology," it is stated that these bulbs are probably photosensitive, despite the fact that Murbach (1907) failed to find any evidence that they are more sensitive to light than other parts of the bell. I have not had any specimens of *G. murbachii* available for sectioning but I have sectioned the tentacular bulbs of *G. vertens*, the Puget Sound species, fixed in Flemming's fluid for the purpose. The sections show no trace of a photoreceptor in the bulbs of this species although there is an abundance of sensory epithelium. In fact, photoreceptors are not known to occur in any of the Olindiidae, the family to which *Gonionemus* belongs. I think it may safely be concluded that the species of *Gonionemus* are devoid of special photoreceptors although these medusae do react to light.

The tentacular bulbs of hydromedusae thus have three important functions: they act as depots for the manufacture and storage of nematocysts, they are organs of general sensory perception, and they are very active in intracellular digestion.

Summary

1. Studies were made in Puget Sound on several medusae belonging to the groups Anthomedusae (*Stomotoca, Sarsia*), Leptomedusae (*Aequorea, Halistaura, Phialidium*), and Stauromedusae (*Haliclystus*).

2. The water content was determined for four species (*Aequorea, Halistaura, Phialidium, Sarsia*) and found to range from 96.5 to 97 per cent (salinity of the sea water 3.09 per cent). Evidence was obtained that the water content declines slightly with increasing size.

3. Activities are limited and responses cannot be elicited with dependable regularity.

4. All species observed have alternate periods of pulsation and floating; in general these periods bear no absolute or relative time duration to each other.

5. Behavior is correlated with morphology and hence shows characteristic differences between the different groups.

6. Mechanical or chemical stimulation, when effective, elicits either an escape reaction or the feeding reaction.

7. The escape reaction may consist of bell pulsations but to stronger stimuli a general contraction, termed crumpling, accompanied by cessation of swimming, is given. *Aequorea* is anatomically incapable of this escape reaction because of its thick stiff bell.

8. The feeding reaction consists in the Leptomedusae and *Haliclystus* of bending the stimulated region of the margin to the manubrium which also moves to meet the inbent margin. In the Anthomedusae it consists of turning the long manubrium in the direction of stimulation.

9. Food was traced through the gastrovascular system by feeding bits of animal flesh coated with carmine. Within half an hour after feeding, food begins to spread along the radial canals and in two or three hours occupies the entire gastrovascular system.

10. The principal sites of intracellular digestion are the lining epithelium of the manubrium, stomach, and tentacular bulbs. Radial, circular, and tentacular canals play a minor rôle. Those portions of the radial canals adjacent to gonads (Leptomedusae) are much more active in intracellular digestion than radial canals elsewhere.

11. No definitely directed flagellar currents could be observed in the gastrovascular system.

12. Strands of carmine-containing mucus were seen to be extruded from the so-called excretory pores of the radial canals (*Aequorea*).

13. The fluid from the stomach of *Aequorea* is usually considerably more acid than sea water (to pH 7.2, mostly 7.4–7.6). It contains a proteolytic and a lipolytic enzyme but appeared to be devoid of diastases.

14. Feeding and escape reactions of *Haliclystus* (a sessile stauromedusan) are similar to those of Leptomedusae. No function could be found for the anchors; the animal gave no response to chemical or mechanical stimulation of the anchors nor were they used for adhesion.

15. The tentacular bulbs of hydromedusae function as depots for the manufacture and storage of nematocysts, as organs of general sensory perception, and as important sites of intracellular digestion. They are not photoreceptors although in Anthomedusae they bear the ocelli when these are present. The tentacular bulbs of *Gonionemus* are devoid of differentiated photoreceptors.

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THE ANAEROBIC GASEOUS METABOLISM OF THE ROACH, CRYPTOCERCUS PUNCTULATUS SCUDDER

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In the experiments of Davis and Slater (1926, 1928) on the anaerobic metabolism of the cockroach, *Periplaneta orientalis,* the oxygen debt incurred during anaerobiosis was paid off exactly, and the excess respiration of recovery appeared to be due to the complete oxidation of the lactic acid produced. I have recently shown (Gilmour, 1940) that in the termite, *Zootermopsis nevadensis,* about half the oxygen debt is repaid, and that the carbon dioxide produced during anaerobiosis is not all due to the buffering of acids.

The general habits and mode of nutrition of the primitive wood-feeding cockroach, *Cryptocercus punctulatus* Scudder, are similar in many ways to those of the lower termites. In common with them, the roach possesses a well-developed intestinal fauna of flagellates, which are responsible for the digestion of cellulose (Cleveland, 1934). It seemed likely, therefore, that a consideration of the anaerobic metabolism of this insect, which in some ways seems to occupy a position intermediate between the higher roaches and the more primitive termites, might yield information on the nature of the differences in the anaerobic processes of the two groups.

Acknowledgments

My thanks are due to Dr. L. R. Cleveland of the Biological Laboratories, Harvard University, for providing the material used in this study and for his interest during the course of the work, to Drs. A. C. Redfield and T. J. B. Stier of the same laboratory for their advice, and to Dr. C. M. Pomerat of Clark University for the loan of a number of respirometers.

Material and Methods

The insects were collected near Blacksburg, Virginia. Two different batches of material were used in the experiments. The first was collected in the fall of 1938 and kept in the laboratory during winter

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at a room temperature of about 22° C.; the second was collected in the spring of 1939 and kept during the summer in a constant-temperature room at 15° C. The diet consisted of moist paper towelling or filter paper.

Respiration was measured by means of Warburg manometers. Two different types of respirometer vessel were used. The first was that described by Pomerat and Zarrow (1937); the other was of the common conical design without centre-well and with a double side-arm. Respiratory quotients were measured by following oxygen uptake for a certain length of time (usually one hour) and then determining the carbon dioxide absorbed, by acidification of the alkali. Fully-grown roaches were studied throughout, one insect being used in each experiment.

Activity was reduced to a minimum by lowering the temperature and placing each insect in a small cage. The cages were made of perforated celluloid, the perforations being sufficiently large and numerous that there was no interference with the diffusion, in and out, of gases. The temperature of the experiments in which the first batch of animals was investigated was 7.5° C. For the second batch, it was necessary to reduce the temperature to 5° C. in order to eliminate all movement. This difference may have been due to the fact that the temperature at which the insects were cultured was lower in the second group than it had been in the first.

Anaerobic conditions were produced by running pure nitrogen (moist and equilibrated to the temperature of the water bath) through the respirometer vessel for a period of 15 to 20 minutes. At the end of the anaerobic period the nitrogen was replaced by CO_2-free air.

The insects were defaunated by exposing them to oxygen at 4 atmospheres pressure for 2 hours.

Experimental Results

Normal Insects

Aerobic Respiration.—The oxygen consumption of normal individuals was 46.1 cu. mm. per gram wet weight per hour at 7.5° C. (average of 70 experiments varying between 23.8 and 68.5), and 28.5 cu. mm. per gram per hour at 5° C. (average of 12 experiments varying between 20.5 and 37.5).

The respiratory quotient varied widely (0.79–1.65), the average of 30 experiments being 1.09. There were no significant differences between the results of experiments run at 5° C. and those at 7.5° C.

The production of combustible gases by the protozoa was investigated in a number of experiments, in which the insects were placed in a closed chamber and the gases present after an interval of some hours analysed with a Haldane apparatus. The protozoa of the roach were found to produce much smaller quantities of combustible gases than did those of the termite. The rate of evolution at 22° C. was about 2 cu. mm. per gram insect per hour—less than 1 per cent of the insect's oxygen uptake, whereas in the termite it has been shown to be about 10 per cent. The analyses indicated that hydrogen was not the only gas evolved, since in almost every case some carbon dioxide was formed as a result of the combustion. It was not possible, however, to arrive at any quantitative estimation of the amounts of different fractions present, since, even when the experiments were continued for such a long time that a large proportion of the oxygen present had been used up, the concentration of combustible gases in the sample analysed was usually less than 0.1 per cent.

This slow rate of evolution of combustible gases was confirmed subsequently in the anaerobic experiments, there being no significant increase in pressure when the respirometers were filled with nitrogen and carbon dioxide was being absorbed by alkali.

The high respiratory quotient is most probably due here, as in the termite, to the presence of large numbers of anaerobic protozoa.

Effects of Anaerobiosis.—When returned to air after 4 hours in nitrogen at room temperature, *Cryptocercus* remained motionless for 1 to 1½ hours, and thereafter showed increasing activity, returning apparently to normal after about three hours. An anaerobic period of 5 hours proved fatal to some, while others recovered, but none survived 6 hours in nitrogen. There was a partial recovery after 6 hours, and even to a smaller extent after 7 hours, but the insects were all dead on the day following the treatment.

Oxygen Debt.—In all the oxygen debt experiments the oxygen consumption was first followed for 2 to 3 hours, after which the air in the respirometers was replaced by nitrogen. After an anaerobic period of 1 hour, the recovery respiration was followed until a steady rate of oxygen consumption had been re-established and maintained for 2 or more hours. The results of these experiments are presented in Table I. The final rate of oxygen uptake is not far removed from the initial rate, but there is, in general, a tendency for the rate after recovery to be higher. The final rate, however, seems to be quite as constant as the initial, and has been followed for as long as 4 hours.

Cryptocercus constantly consumes during recovery an excess amount of oxygen greater than the amount of oxygen missed in anaerobiosis,

and, in fact, repays the debt, on the average, three times over. By "oxygen missed in anaerobiosis" is meant the amount of oxygen which the insect would have consumed during the same period if it had been in air. The time required to effect complete recovery after only 1 hour of anaerobiosis was 4 hours.

Carbon Dioxide Excretion During and After Anaerobiosis.—Experiments on carbon dioxide excretion during anaerobiosis were performed in the same way as for a determination of respiratory quotient,

TABLE I

Repayment of oxygen debt in untreated insects.

The duration of anaerobiosis was approximately one hour in each case. Any slight deviations from this time have been taken into account in calculating the oxygen missed.

Experiment	Temperature °C.	Initial O_2 uptake $mm^3./gm./hr.$	O_2 missed $mm^3./gm.$	Final O_2 uptake $mm^3./gm./hr.$	Excess O_2 consumed $mm^3./gm.$	Excess O_2 consumed / O_2 missed × 100
A 16 V	7.5	50	58	55	187	320
A 16 IV	"	43	50	55	133	270
A 25 I	"	41	48	47	142	300
A 25 IV	"	41	48	54	133	280
A 27 (2)	"	56	70	60	223	320
A 27 I	"	41	48	47	132	280
A 27 IV	"	39	46	52	95	210
A 27 V	"	41	48	56	191	400
A 27 (1)	"	40	50	43	167	330
B 1 (1)	"	57	57	69	106	190
B 1 (2)	"	52	52	38	91	180
Average		46	52	52	145	280
B 2 (i)	5.0	30	30	31	99	330
B 2 (ii)	"	27	27	29	79	290
B 2 (1)	"	23	23	28	80	350
B 2 (2)	"	26	26	29	112	430
B 32 (2)	"	34	34	38	123	360
Average		28	28	31	99	350

except that the air in the respirometer was replaced by a rapid stream of nitrogen before the run was started. The carbon dioxide taken up by the alkali was liberated after an anaerobic period of 1 hour in each case. The results of 4 experiments, all done at 5° C. were as follows:

(cu. mm. per gram) 79 89 78 75 average 80.

The amount of carbon dioxide excreted during anaerobiosis is thus almost three times as great as the amount of carbon dioxide produced during a similar period in air.

TABLE II

Respiratory quotients at successive one-hour periods after anaerobiosis. Untreated insects.

These figures do not represent successive determinations on single insects. Each quotient is obtained from a separate individual.

Hourly periods	1	2	3	4	5	6
	0.31	0.58	0.86	0.90	1.00	1.02
	0.34	0.80	0.84	1.00	0.82	0.92
	0.71	0.78	0.80	0.55	1.18	1.17
	0.51	0.52	0.45	0.55	1.30	0.98
R.Q.	0.46	1.12	1.04	1.05	0.99	1.14
	0.58	0.54	0.77	1.13	1.14	1.00
	0.57	0.75	0.86	0.92	0.97	1.13
	0.55	0.67	0.65	0.96	1.45	1.65
	0.59	0.92	0.97	0.76	1.18	—
	—	0.90	0.71	0.63	—	—
Average	0.51	0.76	0.80	0.85	1.11	1.13

The carbon dioxide retained during recovery was determined by subjecting the insects to nitrogen treatment for 1 hour, and then observing respiratory quotients over 1-hour periods for 6 successive hours. Since the individual variation in respiratory quotient is great, it was necessary to make many different determinations for each 1-hour period

TABLE III

Carbon dioxide retention during recovery. Untreated insects.

Hourly periods	O_2 consumed $mm^3./gm.$	CO_2 produced $mm^3./gm.$	Expected CO_2 production $mm^3./gm.$	CO_2 retained $mm^3./gm.$
1	65	33	69	36
2	72	55	76	21
3	47	38	51	13
4	45	38	49	11
5	31	35	35	—
6	31	35	35	—
			Total	81

in order to obtain significant mean values. The results of these experiments are given in Table II. There is a reduction in respiratory quotient during the recovery period. By the end of 4 hours the quotient has returned to its normal value.

In Table III is shown the carbon dioxide excretion for each hour, calculated from the respiratory quotients and the curve for average

oxygen consumption. The carbon dioxide retained is determined by subtracting the actual carbon dioxide production from the theoretical, the latter being calculated on the assumption that the recovery process consists of an excess respiration having a respiratory quotient of 1.0 superimposed upon the "basal" respiration. The total amount of carbon dioxide retained is 81 cu. mm. per gram, which agrees very closely with the amount of carbon dioxide excreted during anaerobiosis.

Defaunated Insects

Aerobic Respiration.—All experiments on defaunated roaches were carried out at 5° C.

The average oxygen consumption was 44.6 cu. mm. per gram per hour (28 experiments, with results varying between 31.0 and 65.8). There is thus a decided increase over the rate of respiration of untreated insects. A small fraction of this increase is presumably due to a decrease in weight by the removal of anaerobic protozoan tissue. The average weight of defaunated insects was 0.67 gram, that of untreated insects, 0.73 gram.

The average respiratory quotient following defaunation was 0.69 (16 experiments, varying between 0.35 and 0.99). The results, obtained at intervals between the first and fifteenth days following defaunation, showed no definite trend with time.

Oxygen Debt.—The course of oxygen consumption during recovery from anaerobiosis in defaunated roaches is by no means the same as in normal insects. In Table IV are collected the results of experiments on the oxygen debt of defaunated insects.

In the roaches investigated 1 day after defaunation the excess oxygen uptake of recovery is only a little more than the oxygen missed. At 6 days after defaunation the insects repay twice the oxygen debt, while at 8 days the percentage repayment approaches even closer to the value for normal insects. The rate of aerobic respiration remains high in the insects studied at 6 and 8 days after defaunation, as it had been in those at 1 day.

In Fig. 1 the results of all the oxygen debt experiments done at 5° C. are presented graphically, as total oxygen consumed against time. All the results have been equated to the same base-line, and the oxygen consumed per unit time in the recovery period is expressed as a percentage of the initial rate. The graph illustrates the depression of the curve in freshly-defaunated insects and the return towards the normal curve in experiments done at longer intervals after defaunation.

In the course of these experiments a few insects were encountered in which defaunation was not complete. Apparently at the time of

oxygen treatment they had contained cysts,[2] which had not been destroyed. When they were examined at the end of the oxygen debt experiments, the intestine was found to contain numerous cysts, although the number present was much smaller than the normal population of

Table IV

Repayment of oxygen debt in defaunated insects.

The duration of anaerobiosis was approximately one hour in each case. Any slight deviations from this time have been taken into account in calculating the oxygen missed.

Experiment	Temp. °C.	Initial O_2 uptake $mm^3./gm./hr.$	O_2 missed $mm^3./gm.$	Final O_2 uptake $mm^3./gm./hr.$	Excess O_2 consumed $mm^3./gm.$	Excess O_2 consumed / O_2 missed × 100	Remarks
B 18 (i)	5.0	34	37	31	50	140	
B 18 (ii)	"	51	55	58	63	110	
B 18 (iii)	"	32	35	32	45	130	1 day after defaunation
B 18 (2)	"	53	57	48	82	140	
B 29 (ii)	"	59	59	66	73	120	
B 29 (iii)	"	36	36	42	57	160	
B 32 (i)	"	40	40	45	54	140	
Average		44	46	46	61	130	
B 29 (i)	5.0	38	38	40	80	210	6 days after defaunation
B 29 (2)	"	39	39	43	77	200	
Average		39	39	42	79	200	
B 32 (ii)	5.0	37	37	38	111	300	8 days after defaunation
B 32 (iii)	"	44	44	40	101	230	
Average		41	41	39	106	260	
B 18 (1)	5.0	44	48	43	166	350	Unsuccessful defaunation; cysts present
B 29 (1)	"	52	52	66	180	350	
B 32 (1)	"	46	46	53	91	200	
Average		47	49	54	146	300	
B 1 (iii)	7.5	54	54	58	41	80	Untreated; just moulted

active protozoa. These roaches behaved, as regards the repayment of oxygen debt, just as did the normal insects, repaying three times the amount of oxygen missed (Table IV).

[2] True cysts are formed in *Trichonympha*, but the corresponding stage in *Barbulanympha* is a rounded resistant form without a cyst wall. Cf. Cleveland, *et al.* (*loc. cit.*), pp. 236 and 262.

One other insect deserves special mention (Table IV, *B* 1 (iii)). It was an untreated roach, but had undergone a moult just prior to the time of the experiment. This individual showed the lowest percentage repayment of debt of all (80 per cent). Although the contents of the gut were not examined, this insect also presumably contained resistant forms, this being the means by which the protozoa are enabled to maintain themselves from one instar to the next.

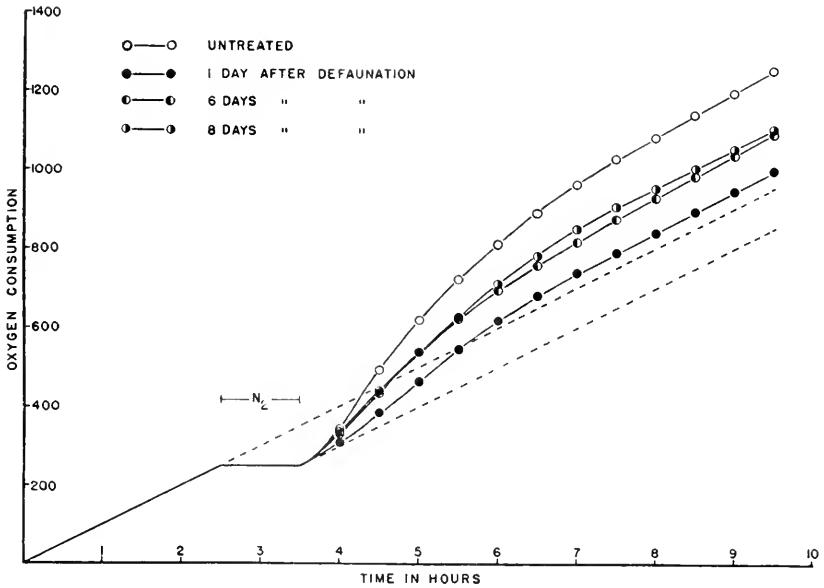

FIG. 1. Repayment of oxygen debt in normal and defaunated *Cryptocercus;* averages of all experiments at 5° C. Ordinates represent total oxygen consumed. The initial oxygen uptake per gram per hour has been made equal to 100 in each group.

Carbon Dioxide Excretion During and After Anaerobiosis.—Experiments on the excretion of carbon dioxide by defaunated insects during 1 hour of anaerobiosis yielded the following results:

(cu. mm. per gram) 84 88 72 52 average 74.

These experiments were done on insects which had been defaunated 7 days previously. The average is not significantly different from the figure for carbon dioxide excretion during anaerobiosis in normal insects. Actually one would expect it to be less than the value for normal insects by an amount equal to the carbon dioxide production of the protozoa. But since the latter would amount to only 5 to 10 cu. mm.

per gram, it would require a large number of experiments to establish such a difference.

A series of determinations of respiratory quotients at successive hours after anaerobiosis was also done on defaunated insects, and the results are presented in Table V. There are fewer experiments than in the series on normal insects and the averages consequently show a less uniform trend. These experiments were carried out either 4, 5 or 7 days subsequent to the defaunation of the insects. The average oxygen uptake of insects investigated 6 days after defaunation was therefore used in determining carbon dioxide excretion, since these figures would approximate most closely to the oxygen uptake of the insects used in the respiratory quotient experiments. The amount of carbon dioxide retained, determined on this basis, is 81 cu. mm. per gram, exactly the same as the carbon dioxide retention of normal insects.

TABLE V

Respiratory quotients at successive one-hour periods after anaerobiosis. Defaunated insects.

Hourly periods	1	2	3	4	5	6
	0.23	0.73	0.72	0.59	0.66	0.83
	0.31	0.44	0.26	0.49	0.57	0.99
R.Q.	0.22	0.64	0.70	0.54	0.66	0.50
	0.19	0.37	0.69	0.39	0.74	0.77
	0.37	0.50	0.81	0.69	—	—
Average	0.26	0.54	0.64	0.54	0.66	0.77

DISCUSSION

It is apparent that the anaerobic gaseous metabolism of *Cryptocercus* differs in almost every particular from that of *Zootermopsis*. In the first place, the carbon dioxide retained in recovery is equal, both in untreated and defaunated insects, to the carbon dioxide excreted during anaerobiosis, which suggests that the latter is derived entirely from the acidification of bicarbonate. It is thus possible that the anaerobic processes in *Cryptocercus* consist entirely of lactic acid glycolysis.

Another point of difference between the two insects is in the percentage repayment of the oxygen debt. Furthermore, there is a decided disagreement in *Cryptocercus* in the amount of repayment between normal and defaunated individuals. A suggestion of minor differences of this nature were obtained from the experiments on *Zootermopsis,* but nothing of the magnitude and regularity of the differences found in

Cryptocercus. It may be stated, broadly, that the removal of *active* protozoa from the gut in some way affects the metabolism so that the percentage repayment of oxygen debt is at first much reduced, although this effect wears off gradually. It is difficult to find an explanation for this phenomenon which would fit all the facts. It seems improbable that defaunation has produced any profound change in the nature of the anaerobic processes, since carbon dioxide excretion during and after anaerobiosis is not altered from the normal.

It is possible that the difference in the repayment of oxygen debt is associated with the increased aerobic respiration of defaunated animals. The oxygen uptake of normal insects is 29 cu. mm. per gram per hour and the carbon dioxide production is 32 cu. mm. per gram per hour, while in defaunated individuals carbon dioxide production is 31 cu. mm. per gram per hour and oxygen uptake is 45 cu. mm. per gram per hour. This suggests that the change following defaunation might have been simply the addition of some process which consumes oxygen without producing carbon dioxide. If this were the case, it would be incorrect to compare the excess oxygen uptake of recovery to an oxygen debt based on the previous aerobic respiration. That there is no change in carbon dioxide production per gram coincident with the removal of the protozoa is not surprising, since there is at the same time a loss of weight representing probably the weight of the protozoa, there being no compensation as there was in the termite, by the addition of an equal weight of non-living material.

The results from insects investigated at 6 and 8 days after defaunation and from those in which cysts were present cannot be explained on these lines, however, since the repayment of the debt was high, in spite of a high rate of oxygen uptake. Perhaps defaunation produced some change in the basal energy requirements in anaerobiosis.

The exaggerated repayment of the oxygen debt in *Cryptocercus* is interesting in the light of the conclusion of Davis and Slater (1928) that in the common cockroach all the lactic acid produced in anaerobiosis is oxidised during recovery, none being resynthesized to glycogen. Davis and Slater found only a fifth of the amount of lactic acid they had expected, and if the rate of aerobic respiration they observed was the actual basal rate, lactic acid glycolysis could have represented only part of the anaerobic processes. Alternatively, if glycolysis were the only mechanism, the aerobic respiration they observed must have been about five times as great as the actual basal value, since an anaerobic mechanism depending entirely on glycolysis, in which all the lactic acid was oxidised during recovery, would entail a repayment of the debt many times over.

The only other attempt to arrive at a quantitative estimate of the repayment of the debt in insects seems to be that of Bodine (1928) on grasshoppers. The duration of anaerobiosis was not very accurately controlled, and the repayment was sometimes less, sometimes more than 100 per cent. Bodine stated that " in carefully-controlled experiments," it could be shown to be approximately equal to the oxygen debt.

If the anaerobic metabolism in *Cryptocercus* is maintained by glycolysis, as the results suggest, the course of the oxygen uptake during recovery means that a large proportion of the lactic acid is being burned. The anaerobic metabolism of *Cryptocercus* thus resembles more nearly that of other roaches than it does that of the termite, which agrees so closely with it in the method of nutrition.

The association of a high carbon dioxide retention with a high percentage repayment of the oxygen debt in *Cryptocercus* and a low carbon dioxide retention (particularly in relation to the anaerobic carbon dioxide excretion) with a low percentage repayment of oxygen debt in *Zootermopsis* suggests that the repayment of an oxygen debt is perhaps, in fact, related specifically to the removal of lactic acid during recovery.

Summary

Fully-grown specimens of *Cryptocercus punctulatus* Scudder had an average oxygen consumption of 46.1 cu. mm. per gram per hour at 7.5° C. and 28.5 cu. mm. per gram per hour at 5° C. The average respiratory quotient was 1.09.

The production of combustible gases by the intestinal protozoa was found to be much less than that previously reported for the termite, *Zootermopsis*.

Complete exclusion of oxygen was fatal to the insects if continued for more than 5 or 6 hours.

The extra oxygen consumed during oxidative recovery from a period of anaerobiosis lasting 1 hour was, on the average, three times as great as the amount of oxygen missed. Recovery was complete at the end of 4 hours after the re-admission of air.

The carbon dioxide excreted during anaerobiosis was 80 cu. mm. per gram in 1 hour at 5° C. and was equal to the amount of carbon dioxide retained during recovery, indicating that the carbon dioxide evolved during anaerobiosis was probably all derived from the acidification of bicarbonate.

In defaunated roaches the average oxygen consumption at 5° C. was 44.6 cu. mm. per gram per hour. The respiratory quotient was 0.69.

In insects investigated 1 day after defaunation the extra oxygen consumed during recovery from anaerobiosis amounted to only 130 per

cent of the oxygen debt. At 6 days after defaunation 200 per cent of the debt was paid off, and at 8 days, 260 per cent.

Insects which had undergone the defaunation treatment, but in which defaunation was not complete, owing to the presence of resistant stages of the protozoa, had a heightened rate of oxygen consumption but behaved like normal insects, paying off 300 per cent of the debt.

The anaerobic carbon dioxide excretion and carbon dioxide retention during recovery of defaunated roaches were the same as those of untreated individuals.

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CELL TYPES AND CLOTTING REACTIONS IN THE ECHINOID, MELLITA QUINQUIESPERFORATA

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Introduction

Most of the investigations of the cellular elements and morphology of clotting in Echinoidea have been done on sea-urchins and heart-urchins (Geddes, 1880; Cuénot, 1891; Kindred, 1921, 1924) with an occasional reference to sand-dollars (Kindred, 1924), but none to *Mellita quinquiesperforata*. It is the object of this work to study the cellular elements in the perivisceral fluid of *Mellita* and to determine the sequence of events in clotting under normal and experimental conditions.

The factors which will induce or inhibit clotting in the blood of vertebrates have been the object of numerous investigations, but little work of this nature has been done on invertebrate blood. The work of Donnellon (1938) on *Arbacia* is the only one which has considered experimentally the factors responsible for agglutination of cellular elements in Echinoidea.

Material and Methods

Mellita quinquiesperforata used in this study were obtained by dredging in the coastal waters of Beaufort, N. C. The supply, renewed every other day, was kept in running sea water in laboratory tanks.

To determine the types of cells present, fluid was drained from the perivisceral cavity, a drop placed on a coverslip, inverted over a depression slide, and examined immediately under oil immersion. Camera lucida sketches were made of each cell type. Cells of each type were measured with an ocular micrometer, and their dimensions calculated from twenty of these random measurements.

In order to study phagocytosis, 1 cc. of finely granulated carmine suspension in sea water was injected by use of a hypodermic syringe through the peristomial membrane into three sections of the perivisceral cavity. After definite time intervals, one-half hour to five days, hanging-drop preparations of the perivisceral fluid were examined under oil immersion for phagocytic cells.

This same method was followed in studying fat ingestion. Hard-boiled egg yolk was ground in a mortar, and 1 cc. of a sea water suspension was injected into each animal. Olive oil was emulsified with sea water and similarly injected. Periods of three to twenty-seven hours were allowed for ingestion. One per cent osmic acid and an alcoholic solution of Sudan III were used as fat stains.

One per cent solutions of all vital stains were made up with sea water and allowed to settle. One cubic centimeter of a 1 : 100 dilution of each was injected orally into isolated animals. Hanging-drop preparations were made after periods of three to twenty-seven hours, and examined to determine which cells had stained.

To follow accurately the sequence of events in clumping, hanging-drop preparations were made and examined immediately after fluid was removed from the perivisceral cavity, and observations were continued until all cells were involved in clumps. Although clumping is a continuous process, for descriptive purposes it was possible to recognize four phases designated as stage one, two, three, and four (see p. 312). These four stages were used as criteria in judging the rapidity of the clumping of cells when salts were added to the perivisceral fluid. All salts used were Merck reagents. These were prepared in isotonic solutions. The hypertonic salt solution was prepared by adding 8 cc. of 2.5 M NaCl solution to 50 cc. of sea water.

Two cubic centimeters of perivisceral fluid were drained from an animal into a watchglass containing ½ cc. of salt solution. The clumping process was observed under high power objective of the microscope, and the number of seconds necessary for the mixture to reach each of the four clotting stages as timed by a stop-watch was recorded. At least 15 trials involving 15 animals were made with each salt solution. Controls on normal fluid were run simultaneously by substituting sea water for salt solution.

Observations

Cell Types

There are four types of amoebocytes and two varieties of leucocytes in the perivisceral fluid of *Mellita*. The amoebocytes are constantly changing shape and crawl about by formation of blunt pseudopodia. They move with a fair degree of rapidity.

Amoebocytes filled with red spherules average 20.7 μ in length and 8.6 μ in width, and are the most common type (Fig. 1). Those with colorless spherules are approximately the same size (22.9 μ long and 10 μ wide), and behave similarly (Fig. 2). Amoebocytes with variously

shaped, scattered, brown particles are present in smaller numbers than the first two types mentioned. They average 15.7 μ in length and 8.6 μ in width (Fig. 3). The brown particles in the cytoplasm exhibit continuous Brownian movement. A fourth type of amoebocyte filled with yellowish-brown spherules sometimes appears (Fig. 4). This type is more apt to be found after the sand-dollars have been kept in the laboratory for several days. Spherules similar to these are sometimes found in leucocytes with lobed pseudopodia.

There are two varieties of leucocytes; one has large hyaline ectoplasmic lobes and a small central granular endoplasmic portion (Fig. 5); the second type is spherical and is composed of many vesicular compartments, but lacks visible pseudopodia (Fig. 6). The first variety is 32.1 μ long and 27.9 μ wide; the second, 28.6 μ long and 27.9 μ wide. In the vesicles of the second variety may be seen a small amount of homogeneous pinkish material and occasionally small scattered granules which move about by Brownian movement.

Phagocytosis

One-half hour after injection of carmine into the perivisceral cavity small amounts of carmine particles are present in the amoebocytes with brown particles and in two types of leucocytes. Carmine is still present in the same cells but in much larger quantities after five days (Figs. 7, 8).

The amoebocytes with red and white spherules are similar in size, shape, and behavior, but differ in color and fat content as indicated by their reaction to osmic acid and Sudan III. It was thought that if an emulsion of egg yolk or olive oil were injected into the perivisceral cavity, amoebocytes with white spherules might be the only cells to ingest fat and they would change into amoebocytes with red or colored spherules. This was not the case; the leucocytes (Figs. 9–11) were the only cells to ingest fat.

Reaction to Vital Stains

Three hours after injection of vital stains cellular elements were examined. Safranin, sodium carminate, and methylene blue give negative results. Neutral fuchsin, however, stains the brown movable granules in the leucocytes red and similar granules in the brown amoebocytes pink. Neutral red stains the granules in both types of leucocytes red, but does not stain the granules of the brown amoebocyte. At the end of this period all cells are still viable and appear normal.

Normal Clotting

No fibrin or gelation of the plasma is observed in the normal perivisceral fluid. When the plasma is separated by filtration from the cells it remains as a clear filtrate and undergoes no visible change. Thus clotting in *Mellita* involves the cellular elements alone; all cell types, however, are involved. The first indication of clumping occurs 78 seconds after the fluid is removed from the perivisceral cavity. This phase, designated as stage 1 (Fig. 12), is characterized by clumping of a leucocyte with lobed pseudopodia either with one or two like cells or with cells of the other types. The chance meeting and adherence of other cells enlarge the clump (Fig. 13) in stage 2. In the third stage the clump becomes larger and more compact; few free cells are seen in the field. This stage is reached at an average of 145 seconds after removal from the perivisceral cavity. Occasionally an amoebocyte in which red spherules have broken down may be seen (Fig. 14*a*). Usually, however, amoebocytes with spherules or amoebocytes with brown granules continue to move about after they enter the clump. Amoebocytes with spherules are frequently seen radiating out from the clump attached only by a narrow stalk (Fig. 14*b*). It may be noticed that all the spherules are near the distal portion of this cell, and it looks as if this amoebocyte is attempting to free itself from the clump. Occasionally amoebocytes do free themselves, but they are usually retained by an adhesive substance of the leucocyte with lobed pseudopodia.

EXPLANATION OF PLATE I

All figures were drawn with aid of camera lucida. × 1053.

FIG. 1. Three amoebocytes with red spherules to show differences in size and shape.

FIG. 2. Amoebocyte with colorless spherules.

FIG. 3. Amoebocyte filled with brown fluid containing scattered brown granules.

FIG. 4. Amoebocyte with yellowish-brown spherules.

FIG. 5. Leucocyte with large clear pseudopodial lobes.

FIG. 6. Spherical leucocyte without visible pseudopodial projections. Compartments may possess granules which exhibit Brownian movement as in the amoebocyte shown in Fig. 3.

FIGS. 7 AND 8. Leucocyte with lobed pseudopodia and spherical leucocyte showing carmine grains near the center of the cells. Taken from animals which had been injected 5 days previously.

FIG. 9. Leucocyte with pseudopodial lobes which has engulfed a droplet of olive oil stained with osmic acid.

FIG. 10. Spherical leucocyte with engulfed droplet of olive oil stained black with osmic acid.

FIG. 11. Leucocyte with lobed pseudopodia which has engulfed a bit of emulsified egg yolk stained orange with Sudan III.

FIG. 12. First stage of clotting: two leucocytes with lobed pseudopodia and an amoebocyte with red spherules.

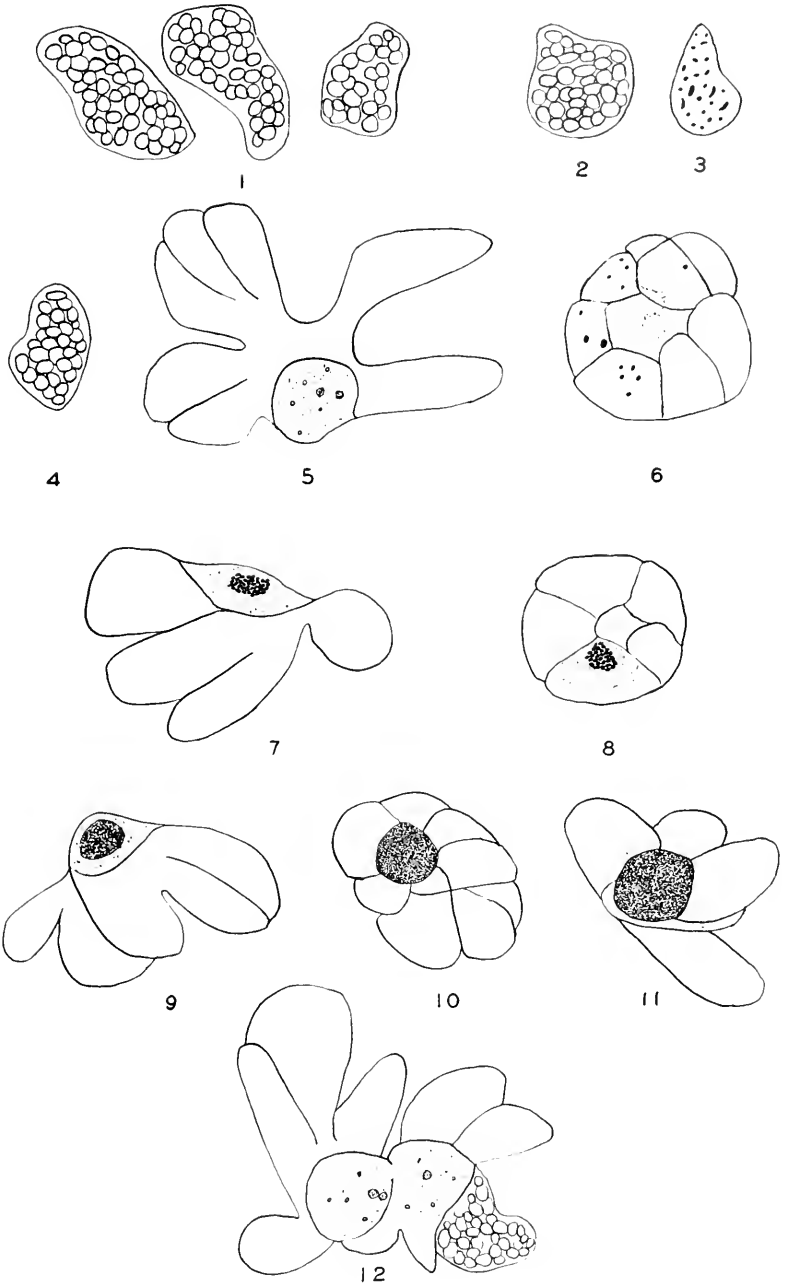

PLATE I

The clumps are large enough to be seen with the naked eye in the fourth stage. They appear as isolated, dark red, opaque masses. Microscopically it can be seen that there is a general breaking down of all cells, especially those near the center of the clump (Fig. 15). The spherules of nearly all the amoebocytes have blended into compact homogeneous masses which often appear trilobed or bilobed before becoming spherical (Figs. 15 *a, b, c*). Only occasionally can an amoebocyte with intact spherules be seen (Fig. 15*d*). The spherical leucocytes are little affected by the general contraction of the whole mass (Fig. 15*e*) but retain their identity. Since no free cells are present, this stage is considered the end-point of clotting and is reached on an average of 165 seconds after removal from the perivisceral cavity.

Action of K and Ca

In an attempt to determine the mechanism which is involved in the clotting reaction of the perivisceral fluid, certain salts were added which might influence the clotting process. The salts used were isotonic solutions of potassium chloride and calcium chloride. These were found to decrease noticeably the time required for clotting (Table I). The results show that the addition of potassium ions decreases the time of clotting much more than does the addition of calcium. When calcium is precipitated by potassium oxalate or rendered slightly ionized by sodium citrate, clotting is greatly inhibited (Table I). Potassium oxalate was more effective than sodium citrate in this respect.

Fat Solvent

A 5 per cent solution of ethyl alcohol decreases clotting time 20.6 per cent. The cells in alcohol solution reached the end-point of clotting

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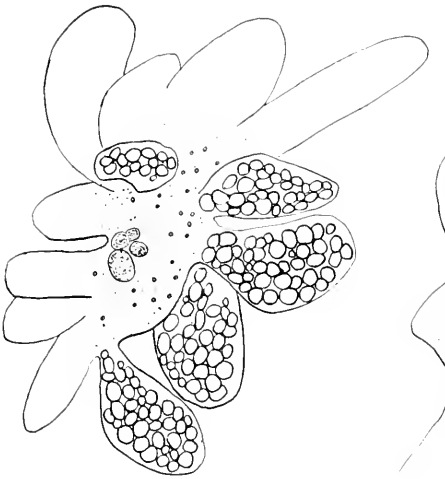
EXPLANATION OF PLATE II

FIG. 13. Second stage of clotting: larger aggregation of cells including leucocytes and amoebocytes with red and white spherules.

FIG. 14. Third stage of clotting: cellular aggregation larger than in previous stage. *a*, amoebocyte in which red spherules have broken down. Cell is filled with red homogeneous material; *b*, amoebocyte with red spherules appears to be attempting to leave the cellular aggregation.

FIG. 15. Fourth stage of clotting. The cellular aggregation has contracted, most of the amoebocytes have become spherical and their spherules broken down into homogeneous masses.

R, amoebocyte with red spherules broken down; *W*, amoebocyte with white spherules broken down; *Y*, amoebocyte with yellow spherules broken down. *a, b, c*, amoebocytes whose spherules have broken down and which now appear as tri-lobed, bi-lobed, or spherical homogeneous masses; *d*, amoebocyte with colorless spherules still intact; *e*, spherical leucocyte unchanged.



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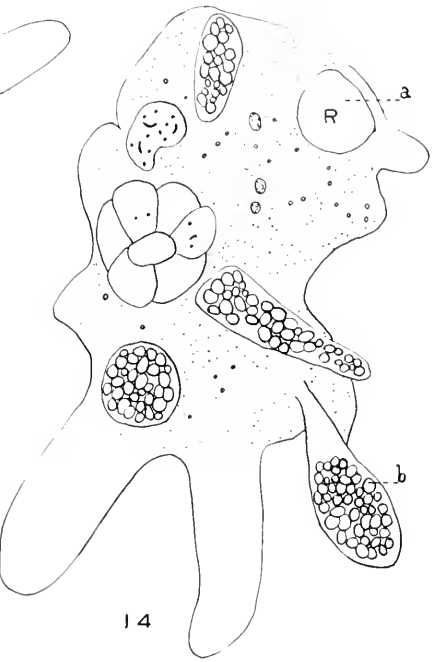

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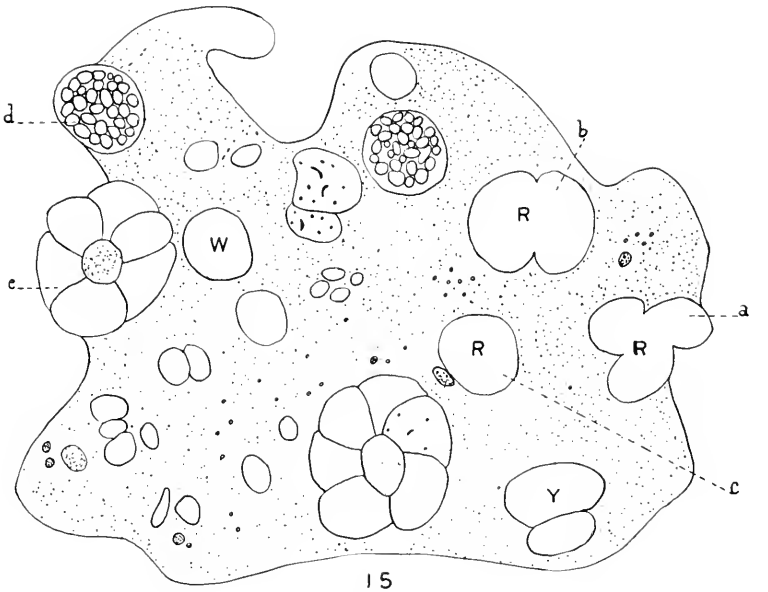

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PLATE II

135 seconds after removal from the perivisceral cavity as compared with 170 seconds in the control.

Hypertonic Salt Solution

A hypertonic salt solution accelerated clotting 45 per cent. The end-point was reached after 95 seconds as compared with 173 seconds in the control.

Action of Magnesium Sulphate

A saturated solution of $MgSO_4$ inhibited clotting indefinitely.

TABLE I

Effect of salts on clotting time of perivisceral fluid of Mellita.

Seconds required for reaction									Percentage Change in Final Clotting Time
Stage of Clotting	First Stage		Second Stage		Third Stage		Fourth Stage		
Salts	with salt	Control	with salt	Control	with salt	Control	with salt	Control	
0.53 M KCl	*		54	90	69	135	77	168	−54
0.3 M $CaCl_2$	66	72	85	89	101	130	117	165	−29
0.27 M Na Citrate	124	69	150	91	181	140	239	171	+40
0.3 M K Oxalate	129	80	151	85	169	142	275	169	+63
Hypertonic NaCl †	63	75	77	80	86	135	95	173	−45

* Too rapid to determine accurately.
† 8 cc. of 2.5 M NaCl to 50 cc. of sea water.

DISCUSSION

Various investigators (Geddes, 1880; Cuénot, 1891; Kindred, 1924) have described amoebocytes with colorless spherules, red spherules, and yellow spherules in sea-urchins. It is interesting to note that in *Mellita* there is a fourth type, a brown fluid-filled amoebocyte with scattered brown granules. This type was not found by Kindred in his comparative study of echinoderms which included the sand-dollar, *Echinarachnius eccentricus*. Geddes (1880), however, described and figured a similar type of cell in the perivisceral fluid of the heart-urchin, *Spatangus purpureus*. Brown amoebocytes as well as two types of leucocytes are phagocytic, as shown by their ability to ingest carmine grains.

They may, therefore, be important in ingestion and transportation of food particles. When the origin of the granules present in the brown amoebocytes as well as similar granules occasionally found in the vesicles of the spherical leucocyte is known, we may be able to determine whether these are food particles which are in the process of preliminary digestion or are excretory granules.

Amoebocytes with red spherules are the most common type in the perivisceral fluid of *Mellita* and Kindred (1924) reports that they are most numerous in *Strongylocentrotus franciscanus,* but less numerous in *Strongylocentrotus drobachiensis* and in *Echinarachnius eccentricus.* In the latter genus (Kindred, 1924) and in *Spatangus purpureus* and *Echinocardium* (Geddes, 1880) amoebocytes with yellow spherules are most abundant. Thus no generalization can be made as to the relative number of each type of amoebocyte in Echinoidea.

There is a theory that red and yellow amoebocytes are carriers of food. Awerinzew (1911) noticed that a variety of *Strongylocentrotus drobachiensis* which lived upon a rock and mud bottom had a greenish-yellow coloring in the skin; another variety which lived among red algae had a red appearance. Injections of ammonia-carmine and India ink revealed that amoebocytes took up these substances and deposited them in the skin. Hence Awerinzew believed that the red color in the skin and in the amoebocytes was the pigment from the red algae. The habitat of *Mellita* is sandy bottoms where there is little if any red algae, and yet the amoebocytes with red spherules are so numerous that they give the perivisceral fluid a reddish tinge. If Awerinzew's conception were correct amoebocytes would take up carmine grains, as he thought; however, this function has been found to be confined to the leucocytes by Kindred (1924) and to the leucocytes and small brown amoebocytes in our work on *Mellita.*

Tests with osmic acid substantiated by Sudan III reveal that amoebocytes with red spherules contain fat, but injection of fatty substances into the perivisceral fluid failed to reveal that it is ingested directly by any amoebocyte.

Cuénot (1891) observed vibrating cells in the perivisceral fluid of all the sea-urchins and heart-urchins he examined. These cells are not present in *Mellita* and Kindred (1924) found none in *Echinarachnius eccentricus.* In this respect the perivisceral fluid of sand-dollars which have been examined differs from that of other Echinoidea.

The presence of two varieties of leucocytes in *Mellita* is not strictly comparable to the condition found by Kindred (1924). The spherical leucocytes ("bladder-amoebocytes" of Théel, 1921) without visible pseudopodia but with vesicular compartments were not found in any

echinoderm examined by Kindred although the leucocytes with large hyaline ectoplasmic lobes present in *Mellita* were found in all classes of Echinodermata he examined. This latter variety normally possesses broad rounded lobes as Goodrich (1919) maintains, and does not normally form long filiform processes as Geddes (1880) and Cuénot (1891) described.

We have considered the spherical leucocyte a different type from the leucocyte with hyaline ectoplasmic lobes although it is possible that they may be different phases of the same cell. They are considered different types not only because of morphological differences but because the leucocyte with lobed pseudopodia is the only one to take part in the formation of a clot.

Clumping of the cellular elements in the perivisceral fluid of *Mellita* is apparently associated with the sticky exterior or adhesive secretion given off by the leucocyte with lobed pseudopodia. Amoebocytes lack this substance because when they make contact with each other they can separate, but if they come in juxtaposition with a leucocyte with lobed pseudopodia they are usually retained even though they attempt to free themselves. Schäfer (1882) described a coagulable material related to mucin which was given off by leucocytes in sea-urchins. He believed this material to be responsible for clumping. Donnellon (1938) thought that the breakdown of the red spherules of the amoebocytes in *Arbacia* liberated something which had an effect upon clotting. This may play some part, but it is very questionable whether it is important in *Mellita* because it does not occur until the end-point is nearly reached. The clot is consummated in *Mellita* on an average of 165 seconds after the perivisceral fluid has been removed from the body. The end-point of clotting in *Arbacia* (Donnellon, 1938) does not occur until 2,760 to 5,300 seconds.

Donnellon (1938) found that certain salts, such as KCl, $CaCl_2$, a hypertonic solution of NaCl, as well as fat solvents decrease the time of clotting, whereas K-oxalate and Na-citrate inhibited clotting in *Arbacia*. Using the same concentrations and molar values, we have been able to confirm Donnellon's findings. The accelerating or retarding effects were in general the same although the lengths of time involved were different. It should be noted, however, that in our work ethyl alcohol was not as effective in accelerating clotting as were the salts. The results indicate that the factors involved in clotting are somewhat similar in the two animals.

When the perivisceral fluid of *Mellita* is first removed there is little indication of clumping which denotes that leucocytes with lobed pseudo-

podia do not have a sticky exterior when in the perivisceral cavity, or if they do it is not strong enough to entrap other types of cells. Why then do these cells become sticky? It is well known that the blastomeres of developing eggs fall apart when grown in calcium-free sea water. The cement which holds the cells together is apparently due in large measure to calcium (Heilbrunn, 1937). Our experimental work indicates that when calcium ions are removed or are slightly ionized clotting is retarded. Thus calcium may be associated with the sticky substance given off by the leucocytes with lobed pseudopodia.

Our experimental work shows that factors other than calcium are important in the clotting process of *Mellita*. Howell (1912) reported that lacerated or wounded tissues emit a substance, "cephalin," which is essential to clotting in vertebrates, and Donnellon (1938) found that tissue extracts hastened clotting in *Arbacia*. In the light of these investigations it seems as though the tissue factor may be important to clotting in *Mellita*. Further experimental work may eventually determine that substances from the tissue factor release calcium from the leucocytes with lobed pseudopodia, render them sticky, and initiate clotting.

We wish to thank Dr. G. W. Wharton for collecting most of the sand-dollars used.

Summary

1. There are two types of leucocytes in the perivisceral fluid of *Mellita*. The first type is characterized by large ectoplasmic pseudopodial lobes and a small granular endoplasmic center. The second type is spherical and is composed of many vesicular compartments, but lacks visible pseudopodia. Both types are phagocytic, but the first type is the only one important in the clotting process.

2. There are three types of amoebocytes filled with spherules: those with red spherules are most abundant, those with colorless spherules are common, and those with yellow-brown spherules are rare. None of these are phagocytic. A fourth type of amoebocyte, smaller than the first three types, is filled with brown fluid and contains small scattered granules which exhibit Brownian movement. This type is phagocytic.

3. Clotting involves the cellular elements of the perivisceral fluid, but does not include the plasma. As soon as the perivisceral fluid is removed from *Mellita* the leucocytes with large pseudopodial flaps produce a sticky substance to which amoebocytes or other leucocytes adhere and finally become entangled into small clumps. As the endpoint is reached there is a general contraction of the clump, entangled amoebocytes become spherical, and their spherules break down. The

end-point of clotting is reached at an average of 165 seconds after the perivisceral fluid is removed from the body.

4. KCl, hypertonic solution of NaCl, $CaCl_2$, and a 5 per cent solution of ethyl alcohol decrease the time of clotting.

5. Na-citrate and K-oxalate increase the time of clotting, and $MgSO_4$ retards clotting indefinitely.

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ANNUAL FLUCTUATIONS IN THE ABUNDANCE OF MARINE ZOÖPLANKTON [1]

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From the time of the earliest oceanographic expeditions, it has been recognized that the open oceans are more densely populated in high northern and southern latitudes than in the tropical belts, and that the richest areas of all are boreal coastal waters. But even in these fertile zones, the abundance and character of the plankton is known to fluctuate from time to time. Perhaps the most obvious change is a seasonal one. During the spring a wave of reproduction commonly follows the winter period of scarcity, and later in the summer there may or may not be a second period of increase. However, the seasonal cycle is not constant from year to year either in the numbers or in the species of animals produced. In certain cases, annual changes of this sort have been thought due to fluctuations in the amount of " foreign " water entering a given area from neighboring regions. As a consequence, plankton studies in recent years have frequently centered on a search for " indicator " species which would reveal the presence of intruded water and give a clue to its origin. Possibilities were foreseen of the practical use of such " indicators " in forecasting the success of the fisheries or even in solving complex hydrographic problems (for a recent bibliography, see Russell, 1939; additional references, Pierce and Orton, 1939; Redfield, 1939; Sømme, 1934; Bigelow and Sears, 1937; Report Newfoundland Fisheries Research Commission, 1932; Frost, Lindsay, and Thompson, 1933; Thompson and Frost, 1935, 1936).

By far the most extensive plankton investigations based on the study of " indicators " of this sort has been undertaken at Plymouth, England (Russell, 1939). Of the results obtained perhaps the most suggestive are derived from the fluctuations in abundance of the two chaetognaths, *Sagitta setosa* and *Sagitta elegans,* since the former has almost entirely replaced the latter in the years following 1931. At the same time, in the Plymouth region, there has been a continued impoverishment of the phosphates in the water, a scarcity of fish larvae, and finally a failure of the herring fishery. Since it has been concluded that " *S. setosa* pre-

[1] Contribution No. 249 of the Woods Hole Oceanographic Institution.

dominates off Plymouth when the easterly element in the Dover Straits current is suppressed and the flow of Atlantic water into the North Sea from the north is strong (Carruthers theory) " (Russell, 1935, p. 328), the unusual conditions in the plankton are believed due primarily to changes in water circulation.

There are, however, examples of fluctuations among the planktonic animals of the Plymouth region which are not so clearly correlated with a mixing of water masses. Thus, the two species of the siphonophore genus *Muggiaea*—inhabitants of warm coastal waters generally—do not appear together in the waters off Plymouth: *M. atlantica* has been taken regularly at the Seven Stones lightship between 1913 and 1925, whereas *M. kochii* replaced it after 1925—at least until 1934 (Russell, 1934). It is suspected that both these species are " indicators " of coastal waters from the south, i.e., the shallower waters bordering on the Bay of Biscay. If this is true, there must be some factor (or factors) other than transport by currents responsible for fluctuations of species such as these which are presumably inhabitants of the same water mass. Such differences would be extremely difficult to distinguish in an area such as Plymouth, which is beset by strong tidal currents as well as by ocean currents.

It is the purpose of this paper to call attention to the occurrence of marked fluctuations in the zoöplankton on the continental shelf south of Cape Cod, a region which is not subject to such irregular mass incursions of " foreign " water as invade the Plymouth area. Such fluctuations as those just cited for the two species of *Muggiaea* may be due in part, at least, to limiting factors as subtle as those controlling the size of the different year classes among fish.

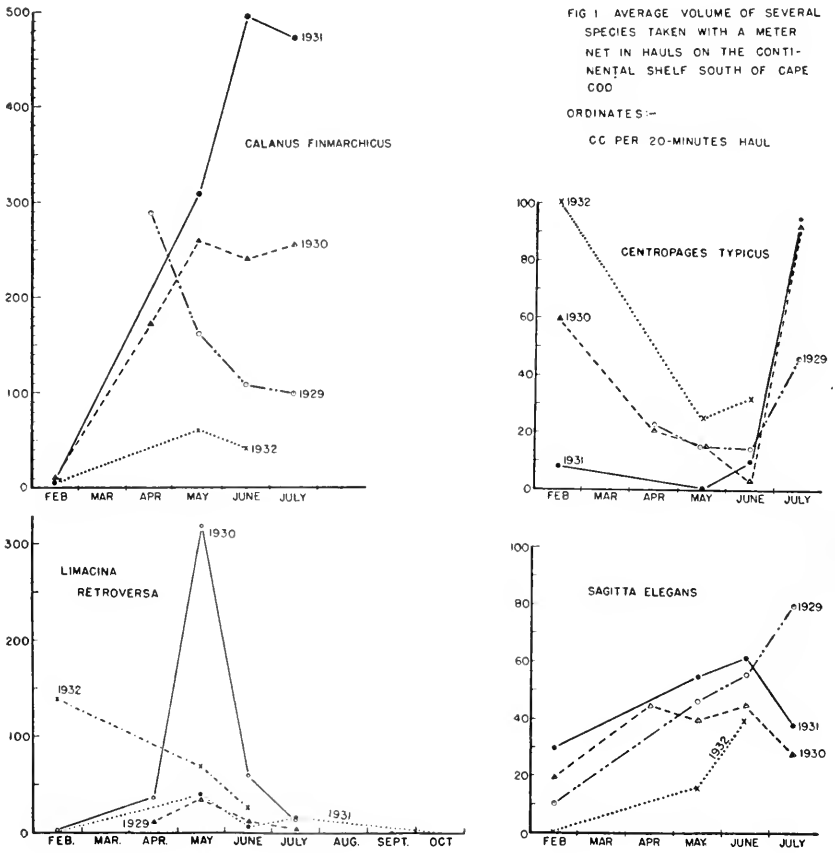
In the coastal waters from Cape Cod to Chesapeake Bay, plankton samples were collected by the U. S. Bureau of Fisheries on 23 cruises during the first half of the years 1929–1932 (Bigelow and Sears, 1939). These observations have been supplemented by stations on a section crossing the shelf off Montauk Point on ten occasions between October, 1937 and June, 1939 (Clarke, 1940) and by collections made at the whistle buoy 3 miles off Martha's Vineyard in 1935–1936 (Sta. 3, Clarke and Zinn, 1937) and on other occasions during the summers of 1937, 1938, and 1939 (unpublished data). These data clearly show important fluctuations not only in the total mass of zoöplankton, but also in the abundance of some of the more common species. Here, the waters undergo essentially the same seasonal changes in temperature and salinity each year (Bigelow, 1933; Bigelow and Sears, 1935; Clarke, 1940) following the cycle which is characteristic of boreal waters generally. A considerable amount of highly saline water from beyond the

edge of the continental shelf must enter this region each year to compensate for the large inflow of river water and rain (Iselin, 1939). However, any transport of plankton into this coastal area by this "slope" water (Iselin, 1936, p. 11, Fig. 2) is slight, since there is always a sharp boundary between the populations of the coastal waters and those offshore. In fact, on the routine cruises now being run SSE from Montauk Point to a point the other side of the Gulf Stream, it is usually possible to sort the catches by casual inspection, according to the locality of capture. The catches on the continental shelf consist essentially of the common coastal species (see Bigelow and Sears, 1939); while those in the "slope" water are composed of *Metridia lucens,* rather than *Calanus* or *Centropages,* as the outstanding copepod, *Sagitta enflata,* as the characteristic chaetognath, and usually salps (*Salpa fusiformis, Iasis zonaria*) in some quantity as well as other typically oceanic species (unpublished data; Clarke, 1940). While *Metridia,* it is true, occurs in some numbers over the outer half of the shelf, particularly in the north, we seldom find other oceanic species more than 10–15 miles inside the 200-meter contour.[2] Hence, while there is some possibility of "contamination" by offshore animals in the region under consideration, it seldom affects any considerable area and never for any length of time.

In addition to the increments derived from the "slope" water, our coastal area also receives water in small quantities from the vicinity of Nantucket Shoals, particularly during the spring. We have little reason to suppose, however, that this has any marked effect on the zoöplankton, for during the spring of 1932, when several intrusions of colder water from the north were observed (Bigelow, 1933), the only noticeable effect of this water movement was the introduction of a meager number of *Calanus hyperboreus* and *Oikopleura labradoriensis* into the area south of Cape Cod (Bigelow and Sears, 1939, p. 247). Actually, during the spring, when flooding from the north most often occurs, the chief effect would be a dilution, since at this season the population in the waters of the Gulf of Maine is at its minimum (Bigelow, 1926). Were there to be an intrusion from this source later in the summer, it is unlikely that the nature of the plankton would be appreciably affected, since catches made in September (1939) on the western part of George's Bank and over the continental shelf between Montauk Point and Martha's Vineyard (unpublished data) were of much the same richness and type, chiefly *Centropages typicus.*

[2] The waters may become "overridden" temporarily with warm-water species which reproduce rapidly (i.e., *Salpa fusiformis*—July 6 to mid-August, 1929). But the enormous quantities of these (3,000–5,000 cc. per 20-minute tow with a meter net) are due to local propagation and are not an indication of an unusually large mixing of "slope" water, because other oceanic species occur only as traces.

Nevertheless in this region between Cape Cod and Chesapeake Bay—an area biologically isolated from the "slope" water offshore and only slightly contaminated by the plankton of Nantucket Shoals—*Calanus finmarchicus* showed a 10-fold fluctuation in abundance (by volume) at the season of its greatest richness during the years 1929–1932 (Fig. 1). In addition, when the richest of these periods (July, 1931) is com-



pared with the very poor periods of a few years later (July 1937–1939), a 600-fold difference (by volume) is revealed. Equally great fluctuations in the stock of *Calanus* have been encountered south of Woods Hole in other years when no extensive surveys were undertaken. This copepod was virtually unobtainable in the vicinity of Woods Hole during the past three summers, although it was abundant during 1935 and 1936 (Clarke and Zinn, 1937).

TABLE I

Species	Year of greatest abundance by volume	Year of least abundance by volume
Calanus finmarchicus	1931	1932
Limacina retroversa	1930	1931
Sagitta elegans	1929, 1931	1932
Centropages typicus	1932	1931
Aglantha digitale	1932	1931
Euthemisto compressa	1930	?

Other individual species studied quantitatively during 1929–1932 have similarly undergone marked fluctuations (more especially over the northern half of the area), notably *Limacina retroversa* (12-fold, Fig.

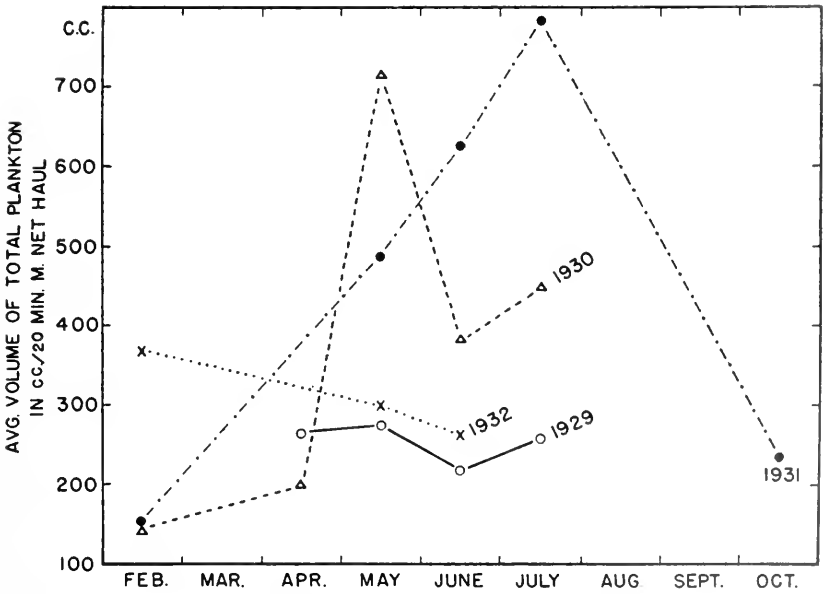


FIG. 2. Average volumes of total plankton.

1), and to a lesser degree *Sagitta elegans* and *Centropages typicus* (Fig. 1). Other members of the plankton which ordinarily occur as odd specimens may become sufficiently numerous in certain years to form a measurable proportion of the total. Such was the case for *Euthemisto compressa* in 1930 and *Aglantha digitale* in 1932. Furthermore, it ap-

pears that these fluctuations occur independently of one another—a year (1931) rich in *Calanus* being poor in *Limacina* or a year (1932) rich in *Centropages* being poor in *Calanus,* etc. (See Table I.)

Not only do individual species exhibit these marked fluctuations, but in addition the plankton as a whole varies in abundance from year to

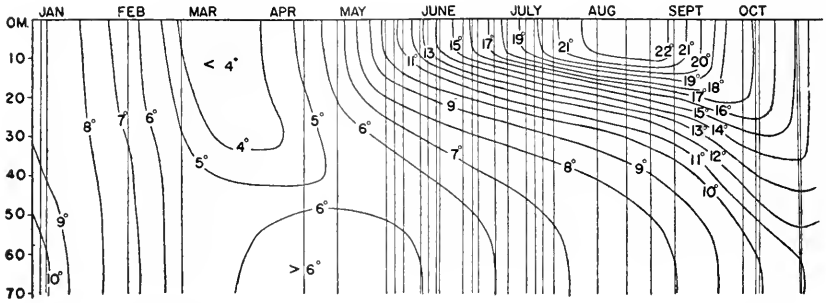


FIG. 3. Average temperatures throughout the year on the continental shelf off Montauk Point, based on all available data.

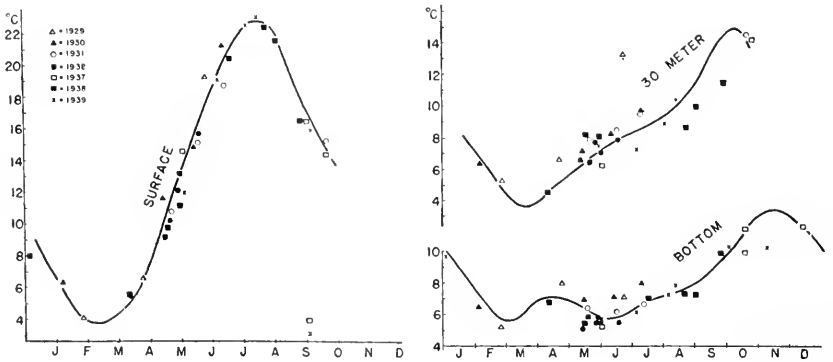


FIG. 4. Departures of temperature from the average seasonal cycle at the surface, 30 meters, and the bottom, for 1929–1932 and 1937–1939, at stations halfway across the continental shelf off Montauk Point.

year (Fig. 2). In the relatively short series of observations (1929–1932), plankton was much more plentiful during the warm winter of 1932 than in the other winters, especially in the south (Bigelow and Sears, 1939), and this condition seemingly continued through late April (judging from a few vertical hauls of about 400 cc. per 20-minute tow—unpublished data). By May and June of that same year, however, the

plankton was distinctly sparse—in fact, as sparse as in 1929. On the other hand, although the plankton had been very poor in the winter of 1931, it was decidedly richer than average during every other month of that year when surveys were made. Earlier surveys had likewise shown similar fluctuations, the plankton being unusually abundant during the cold summer of 1916 and scarce during the warm summer of 1913 (Bigelow, 1926).

Without a longer series of observations we can offer little explanation for these fluctuations. It has been suggested that temperature is the factor in our area which prevents *Calanus* from entering the upper water layers (15–22° C.) in the summer months and restricts this species to the deeper water (7–12° C., Fig. 3). Nevertheless, it does not appear likely that temperature *per se* causes these annual changes in abundance of *Calanus* since the temperature differences are only 1–2° C. between "warm" and "cold" years (Fig. 4)—a difference which would hardly limit the distribution of an animal living over such a wide temperature range as *Calanus*.

We have also made a cursory examination of the salinities and found them to be somewhat more variable locally than the temperatures, from year to year, and even from month to month, especially in the surface layers. However, there appears to be no abnormal mixture of offshore water, the variability seemingly being caused by irregularities in the local rainfall and the inflow from the rivers.

Since the occurrence of wide fluctuations in the abundance of zooplankton in our coastal area now seems well established, further investigation of the consequences of such variation is called for. It is to be hoped, for instance, that a correlation may be found between the richness of the plankton and the fluctuations in the abundance of a commercially important fish such as the mackerel (Sette, 1940), which feeds upon the plankton.

Summary

The abundance of the zoöplankton in the waters overlying the continental shelf between Cape Cod and Chesapeake Bay is shown to be subject to severe annual fluctuations in respect both to individual species and to the total population. Yearly variations of this sort in British waters have been attributed to mass incursions of water from outside sources; but since no great annual differences in the exchange of water masses have been found in the coastal regions treated in this study, our data strongly indicate that important fluctuations in the plankton may occur in relatively undisturbed areas. These changes in the plankton

are not found to be correlated directly with gross environmental changes and therefore must be due to an indirect action of physical or biological factors.

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HETEROPLASTIC TRANSPLANTATION AND SPECIES SPECIFICITY

I. A Comparison of the Effects of Reciprocal Chorio-allantoic Transplants of Macerated and Unmacerated Duck and Chick Kidney Tissue

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Heteroplastic transplantations, especially those designed to study the development of species specificity in the embryo, have often led to results of doubtful significance. Frequent contradictions appeared, and interpretations resulted in ambiguities. As a consequence, any correlation between the transplantability of a tissue and species specificity has been questioned. To study this, and contingent problems, a series of transplantation experiments has been planned using hosts and donors of a stage in development when the relation in question first becomes apparent, and presumably, therefore, when it can be most easily analyzed.

In investigating some of the various aspects of heteroplastic transplantations, Sandstrom and Kauer (1933*a*) studied the host reactions incited by macerated duck kidney tissue implanted on the chorio-allantoic membrane of the chick. They discovered that macerated tissue from the early embryo, e.g., 13 days of incubation, could grow and differentiate, and to a certain degree reconstitute itself into what appeared to be an integrated kidney tissue, unhampered by, and without effect on, the host. But as the donor embryo approached the hatching stage, not only did the macerated kidney tissue fail to grow, but there was evidence that it had a lethal effect on the host. While this drastic effect might have developed gradually, it expressed itself rather suddenly at hatching. Accordingly, implantations of macerated kidney tissue from just-hatched ducklings caused a mortality rate of 100 per cent. They attributed the death of the hosts to an anaphylactoid shock brought about by certain species specific qualities which the protoplasm of the donor tissue apparently acquired just previous to the time of hatching. The results were so striking that a more complete investigation, with an attempt to relate the findings to the results of experiments involving intact tissue transplants, seemed desirable.

A small portion of the results was reported in abstract by Sandstrom, Eisen, and Siffert (1939).

Experimental Procedure

Two series of heteroplastic transplantations were made, namely, duck to chick, and chick to duck. In addition, homoplastic transplants were made to both the chick and the duck. Single-combed White Leghorn chickens and White Pekin ducks furnished both donors and hosts. The ages of the chick donors were selected as follows: embryos of 16 and 20 days' incubation, 5-day old chicks, and adults of uncertain age, while those of the duck donors were embryos of 13, 21, 24, and 27 days' incubation, and ducklings, 1, 4, and 7 days old. The chick hosts were embryos of 9 days' incubation, and the duck hosts, of 13 days' incubation.

In all of the transplantations, tissue from the metanephric kidneys was used. The organ was dissected free from adjacent tissues, and kept in normal saline solution at 38° to 39° C. until a sufficient number of metanephroi of any one stage had been obtained. The time required to collect them varied according to the age of the donor, but usually, at most, it was a matter of only a few minutes. The metanephroi were then completely macerated in a small quantity of saline solution by grinding with a pestle in a glass mortar. After this vigorous treatment the contents of the mortar were washed into a centrifuge tube with saline solution, and the ground tissue centrifuged out. By the technique characteristic of chorio-allantoic grafting, a bit of the collected tissue, only 1 cubic millimeter, was implanted on the chorio-allantoic membrane of the host which was then returned to the incubator for continued incubation.

Examinations of the hosts were made at set intervals in various ways. Usually they were examined at some interval within 48 hours after the implantation, either by candling, noting the condition of the inner shell membrane through an opening into the air chamber, or opening the egg and sacrificing the embryo if it were still alive. In some cases the embryos were to be utilized for another type of experiment, and only a few eggs were examined at any one time, so that the observations extended over several days. Whenever such variations in the procedure might have some bearing on the interpretations of the results, they will be mentioned specifically.

In comparing the results obtained after the implantation of macerated, with those of intact tissue, data from other experiments were utilized for the latter. For this reason the ages of the donors of the intact tissue do not always agree with those of the macerated tissue. The deviation, however, is not so great as to offer any serious objections.

Perhaps it is well to point out here that the mortality rate of the developing chick hosts, which forms the basis of this report, might readily be affected by many factors, some of which are undoubtedly the result of operative procedure, although there is reason to believe that the latter is not of considerable consequence. There are in addition some factors which, under ordinary circumstances, are beyond the control of the experimenter. Reference is being made, of course, to the hardiness of the poultry stock, the handling of the eggs, weather conditions in transit, etc. While a check had been made with respect to the source of the eggs, yet there have been instances when comparable ex-

TABLE I

Mortality rate following implantation of macerated kidney tissue

Host-donor relationship	Number of cases	Age of donor	Age of host	Length of time on host	Number dead	Mortality rate
		(in days)	*(in days)*	*(in days)*		*per cent*
Duck-on-chick	14	13	9	2	3	21.4
	56	21	9	2	21	37.5
	28	24	9	1.25	7	25.0
	27	27	9	2	16	59.2
	57	28	9	1.5	50	87.7
	19	32	9	2	19	100.0
	103	35	9	2	103	100.0
Chick-on-duck	31	16	13	2 to 9	14	45.1
	27	20	13	2	14	51.8
	63	26	13	2	33	52.3
	29	Adult	13	2	8	27.5
Duck-on-duck	39	35	13	2	11	28.2
Chick-on-chick	38	26	9	2	9	23.7

perimental treatment was afforded two separate shipments of eggs, but with quite contrasting results. Although no results were considered when there was reason to suspect unfavorable treatment of the eggs that might affect the viability of the embryos before incubation, e.g., sudden extreme changes in temperature while the eggs were in transit from the hatchery to the laboratory, nevertheless, the percentages which express the mortality rate will show, particularly when the numbers are low, considerable variation which must pass unexplained.

RESULTS

The results of the heteroplastic transplantations are summarized in Table I. Perusal of the table reveals that the macerated duck tissue

had little effect on the chick hosts until the age of the donor material approached that of the hatching period, i.e., the twenty-seventh day of incubation. At this time, the day before hatching, the mortality rate among the hosts, resulting from the implantation of the macerated duck tissue, increased to 59.2 per cent. When the macerated tissue was obtained from donors on the day of hatching, there was an increase in the death rate following implantation to 87.7 per cent, and from older stages of donors it was consistently 100.0 per cent. All the deaths occurred within 48 hours from the time of the implantation. It is an escapable fact that some of the embryos died from causes unrelated to the experiment, but the marked effect on the hosts produced by tissue obtained from donors of hatching age, or older, was most decidedly demonstrated.

In the hosts which survived long enough to permit incorporation, a gross and microscopical study of the chorio-allantoic membrane bearing the implanted tissue indicated some rather definite changes from the normal. Most pronounced was the packing, and apparent agglutination, of the erythrocytes in the blood vessels, both arteries and veins. These vessels in some instances were distended as though by an internal pressure. Diffuse blood clots not infrequently marked the area of implantation, and hemorrhages were also found in the embryo itself. The hemorrhages were for the most part subcutaneous, but some were localized in the hind brain.

While the heteroplastic transplantations of macerated duck kidney tissue displayed certain well-defined effects on the chick hosts, the reciprocal relations did not. As indicated in Table I, the macerated metanephric tissue from the chick, regardless of its age, when implanted on the chorio-allantoic membrane of the duck, had no significant effect on the hosts. To be sure, the death rate for the 16-day donor was not actually as great as indicated, for, as given, the percentage includes all of the deaths that occurred through a period extending from 48 hours to 9 days. While it is possible that the mortality rate at the end of the second day could have been equal to that given in the table, this was not true because the degree of development of some of the host embryos was greater than that of 11 days. The significance, however, of what thus might have been interpreted as an increase in the mortality, is immediately dissipated by the pronounced decrease following the implantation of tissue from the adult chicken. Further evidence that no reaction comparable to that resulting from the implantation of macerated duck tissue occurred, was corroborated by a histological examination of the host membrane. It showed no packing of the blood vessels, nor were diffuse blood clots present in the membrane to the extent that they

were in the other group of heteroplastic implantations. Likewise, brain and subcutaneous hemorrhages were lacking.

The two series of homoplastic transplants involving hatched chicks and ducklings indicated no effects on the hosts. The results, as summarized in Table I, compare favorably with those of the chick-on-duck implantations, and duck-on-chick before the donor had approached the hatching stage.

TABLE II

Mortality rate following implantation of intact kidney tissue

Host-donor relationship	Number of cases	Age of donor	Age of host	Length of time on host	Number dead	Mortality rate
		(*in days*)	(*in days*)	(*in days*)		*per cent*
Duck-on-chick	84	9	9	2 to 9	32	38.1
	94	13	9	2 to 9	46	48.9
	22	19	9	2 to 9	12	54.5
	19	24	9	2 to 9	13	68.4
	43	27	9	2 to 9	16	37.2
	39	Adult	9	2 to 9	4	10.3
Chick-on-duck	41	9	14	2 to 11	6	14.6
	24	13	14	2 to 10	3	12.5
	42	19	14	2 to 10	3	7.1
	16	24	14	2 to 10	9	56.2
	28	27	14	2 to 10	3	10.7
	39	Adult	14	2 to 9	13	33.3
Duck-on-duck	76	13	13 to 14	2 to 10	30	39.4
	36	21	13	2 to 10	10	27.7
	17	24	13	2 to 10	7	41.1
	14	27	13	2 to 10	7	50.0
	27	32	14	2 to 10	9	33.3
Chick-on-chick	62	9	9	2 to 9	18	29.0
	41	19	9	2 to 9	16	39.0
	55	24	9	2 to 9	24	43.6

The effects of the heteroplastic transplantations of the intact, unmacerated kidney tissue are summarized in Table II. The data were compiled from many experiments performed over a period of years, but the general experimental conditions were precisely the same as for the implantations of macerated tissue. The mortality rate, as expressed in this table, however, is not that present after 48 hours, but the total after various periods extending from 48 hours to 9 days. Since many of the hosts showed development beyond the 11-day stage, the percentage that might have expressed the mortality rate at the end of 48 hours can always be assumed to be somewhat less than that recorded. Examina-

tion of the mortality rate shows no significant differences between the various age levels. The same is true for the reciprocal series of chick-on-duck implants. Both sets in the heteroplastic series compare favorably with those in the homoplastic.

Discussion

The results of the implantation of macerated duck tissue to the chick membrane are significant for an understanding of many problems involved in the development of species specificity in the embryo. They emphasize the virtually complete independence of those factors governing the transplantability of a tissue and certain intracellular factors which may be responsible for species specificity, and which become effective only when cell boundaries are broken down. This fact becomes more apparent by a comparison of the effects that macerated duck kidney tissue had on the chick hosts, as included in Table I, with the effects of comparable intact tissue, as summarized in Table II. In the latter group there was no increase in the percentage of deaths following the implantation of tissue from donors of the hatching age, or older. The intracellular factors which were responsible for the effects produced with macerated tissue, although present, were confined within the cell boundaries, and could not, therefore, demonstrate their presence. Furthermore, a comparison of the duck-on-chick transplants of macerated tissue with homoplastic transplants from comparable donors, suggests that the effects on the hosts may be due to factors of importance in species specificity.

Pursuant to the above, it is clear that the problems related to the development of species specificity cannot be adequately analyzed by the utilization of intact tissue transplants only. There is no question that the reaction incited in the tissues of the host by the presence of a bit of engrafted tissue is a response to metabolic substances of a toxic nature, as postulated by Loeb (1930). In other words, it is a typical, localized inflammation reaction. While it is conceivable that a relation exists between the kind and intensity of such reactions and the genetic relationship between the donor and recipient, as contended by Loeb (*loc. cit.*), and further substantiated by Loeb and King (1935), modifying factors are so numerous that they tend to obscure, or confuse the real expression of these relations. For example, Sandstrom (1932) obtained necrosis of intact metanephric tissue of the duck in chorio-allantoic grafts on the chick. The necrosis was preceded and accompanied by a typical cellular reaction. On the other hand, Sandstrom and Kauer (1933*b*) found that cartilage from donors of the same species and of comparable, or older stages, displayed no necrosis, and little

cellular reaction from the chick membrane. Although the species specificity factor was unchanged in the two series of transplants, and therefore should have called forth similar reactions, such was not the case. The nature and greater intensity of the reaction against the actively functional metanephric implant was presumably due to a greater production of toxic substances which produce a proportionately greater reaction in the surrounding host tissue. This response interfered with complete incorporation and vascularization, and ultimately led to an absorption of the transplant and its replacement by connective tissue. In contrast, the passively functional cartilage produced relatively little toxin, and the incorporation of the avascular tissue, rapidly accomplished by the mere contact with the host connective tissue, was unhampered by any reaction which might be antagonistic to a foreign tissue.

That the local reaction can be modified in other ways was also demonstrated by Sandstrom (1934), who obtained functional duck kidney tissue equivalent to that of a 2-day duckling from the chorio-allantoic membrane of the chick after it had grown thereon for a period of 11 days. This condition was possible only because of ideal circumstances wherein the implant was properly incorporated and vascularized before local reactions could interfere. As a result, the toxins of the transplant were removed by the functional graft itself. There was, under these circumstances, no evidence of a host reaction. The most ideal conditions for the establishment of a functional transplant which might grow unhampered by the host would be the concomitant growth and differentiation of the transplant and host. Such an ideal was most successfully achieved by Milford (in press), who obtained normal functional duck kidney tissue equivalent in age to 35 days' incubation after having grown as an intracoelomic graft in the chick for a period of 18 days. The storage of excretory products in these instances offers no difficulty because of the characteristic nature of the excretory function in the bird where uric acid is deposited in the cavity of the allantois after the reabsorption of water by the allantoic membrane. The amount of concentrated uric acid produced by a small graft of kidney tissue after the removal of the water would be relatively small, and readily stored in tissue spaces within the host membrane. As pictured by Sandstrom (*loc. cit.*), it is possible for large kidney tissue grafts to be connected directly by means of their tubules to large vesicular-like spaces which facilitates waste removal. Inasmuch as these and many other factors, will modify the local inflammation reaction, considerable care must be exercised in using transplantations for the study of species specificity, and the resulting conclusions, therefore, must of necessity, be very general.

The violent reaction in the chick host caused by the macerated duck tissue was not a localized manifestation, but was distributed widely in the embryonic system through the blood vascular system. The microscopic and gross examinations of the hosts and their membranes revealed pronounced effects on the blood vascular system not only in the area of the implant, but also in the embryo itself. It could not, of course, be ascertained whether these visible effects were primary or secondary, but the general indications were that the implanted material contained substances which caused an agglutination of the blood cells, and led ultimately to the collapse of the respiratory system. Death was therefore presumably the result of suffocation. It is impossible to state whether this phenomenon of agglutination is in any way related to an immunological reaction, perhaps, in some manner, to the development of antigenic properties and antibody production in the developing embryonic donor and host respectively. Only preliminary attempts have been made to determine quantitatively the minimum amount of macerated tissue necessary to produce death. The effective quantity must be very small, because, of the 1 cu. mm. of macerated tissue implanted, only a small part comes in immediate contact with the membrane. Furthermore, in a few preliminary experiments, it has been found that as little as .3 cc. (5 drops) of the unfiltered, supernatant fluid left after centrifuging macerated kidney tissue from a duckling 4 days old, was sufficient to kill the host in less than 30 hours, but none died before 8 hours. In the transplantation of intact kidney tissue pieces of the metanephros were implanted because the entire organ was too large for proper incorporation. It follows that some of the cells must have been injured by cutting, and the question arises whether the release of the intracellular substance, since the effective dose is so small, did not in some manner bear on the transplantability of the intact tissue. Since care was taken to cause a minimum amount of injury, the amount of intracellular substance freed apparently was insufficient to have demonstrable results, although a comparison of the duck-on-chick with the chick-on-duck transplantations of intact tissue (see Table II) reveals in general a lower mortality rate for the latter group than the former, thus simulating the effects of macerated implants. The significance of this difference is questionable, particularly since the percentages are higher in all age groups, and kidney tissue from duck embryos of 27 days' incubation, and even adults, caused no greater percentage of deaths than did the implants from younger duck donors.

While the results of the implantation of the macerated duck kidney tissue to the chorio-allantoic membrane of the chick have been the source of many interesting speculations and additional problems, the implants

of the macerated chick tissue to the membrane of the duck had no effect on the hosts. Such a complete lack of reciprocity is not without precedence, and in this instance was not wholly unexpected. Sandstrom (1936) demonstrated a decided difference between the results of reciprocal chorio-allantoic transplantations of intact metanephric tissue of the duck and chick. A comparison made between the two types of reciprocal implants, i.e., intact and macerated, tends to confound rather than clarify the problems involved. Whereas the macerated duck tissue at certain ages had profound effects on the chick host, the intact tissue of comparable ages, and even from adults, was rapidly incorporated. In the reciprocal host-donor relationship, in which the macerated tissue had no effect, the intact tissue was only slowly incorporated. No explanation is attempted for these seemingly paradoxical results, but they do give emphasis to the contention that transplantability is independent of intracellular properties.

A comparison of the results of the transplantation of intact and macerated tissue of the duck to the embryonic chick host also displays a striking parallelism to the results of experiments which have attempted to correlate transplantability with serological blood properties of the host. Several workers have compared the success of skin grafts to serological relationships. Baldwin (1920) and Kubanyi (1924) support the conclusions of Masson (1918), who seems to have demonstrated that skin implants could be successful only if the donor was of the same blood group as the host. Others, particularly Kozelka (1933), found evidence to the contrary. He made detailed investigations of the problem, transplanting such integumentary structures of the chicken as the wattle, comb, and spur. He found no relation between the success of the transplant and the agglutinating phenomenon of the host's blood. In the assumed parallelism, the integumentary grafts of Kozelka can be compared to the intact kidney tissue. Any apparently antagonistic reaction would be local, and a response of the host's tissue to the metabolic toxins given off by the transplant. It would therefore be independent, or nearly so, of the specificity of either the host or donor. The agglutinating factors of the blood are at least analogous to the intracellular factors released by crushing the metanephric tissue. Both have lethal effects on the host, but are potent only when, and if, they gain entrance into the blood stream.

Summary

1. Metanephric tissue from duck and chick embryos of comparable ages, ground in a mortar and collected by centrifuging, was implanted on the chorio-allantoic membrane of the chick and duck respectively.

2. The implantation of the macerated duck tissue had no significant effects on the chick hosts until the donor embryos approached the age of hatching, at which time it caused death within 48 hours. The average mortality rate following the implantation of tissue from donors of 24 days' incubation, or younger, was 27.9 per cent. When the tissue was obtained from donors of 27 days' incubation, the percentage of deaths among the hosts increased to 59.2 per cent, and when obtained from donors of 28 days' incubation (time of hatching), it increased to 87.7 per cent. Macerated tissue from donors older than 28 days' incubation consistently killed the hosts, the mortality rate being 100.0 per cent.

3. The reciprocal relation, i.e., chick-on-duck, showed no significant increases in mortality rate comparable to that demonstrated in the duck-on-chick implantations.

4. Death of the chick hosts following the implantation of macerated duck tissue resulted from an apparent agglutination of the blood cells by intracellular substances which were released from the cells by maceration.

5. Comparing the effects of the implantation of macerated tissue with those of the intact, it was concluded that the transplantability of a tissue, as manifested by the nature and intensity of the local inflammation reaction incited by metabolic toxins given off by the transplants of intact kidney tissue, is independent of intracellular substances which may be responsible for species specificity, and which are released from the cell only by crushing.

6. Attention was called to the fact that the local inflammation reaction incited by intact tissue transplants can be modified in several ways, so that it can be used only with considerable care in an analysis of problems pertaining to the development of species specificity.

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1940

JULY 9

Interrelations between egg-nucleus, sperm-nucleus and cytoplasm of the Asterias egg. Robert Chambers and Edward L. Chambers.

Asterias eggs removed from the ovary are in the germinal vesicle stage. Normal maturation occurs within an hour in sea water. Germinal vesicle eggs were cut into nucleated and non-nucleated fragments. These and whole eggs, after fading of the germinal vesicle, were inseminated at intervals before and after polar body formation.

The results obtained were as follows: (1) Insemination before polar body formation accelerates the budding off of the polar bodies by about 10 minutes; (2) The sperm aster never appears until after the second polar body has formed, irrespective of the insemination time; (3) The egg undergoes three distinct maturation phases after the germinal vesicle has faded.

During the first phase (20 to 60 minutes) the sperm nucleus lies quiescent in the egg cytoplasm. During the second phase (60 to 70 minutes) the sperm nucleus develops very slowly. The third phase extends from 70 minutes (shortly after first polar body formation) during which the sperm-nucleus develops at maximal speed.

The series of events may be summarized as follows: The spermatozoön enters the egg and hastens the maturation of the cytoplasm. The cytoplasm reaches complete maturation shortly before the formation of the first polar body. The matured cytoplasm simultaneously allows both egg-nucleus and cytoplasm to start development—on the one hand the polar bodies are pinched off, on the other hand the sperm-aster develops. In the insemination experiments with the nucleated and non-nucleated fragments of the germinal-vesicle egg it was found that the sperm-aster in the non-nucleated fragment develops slightly but constantly earlier than it does in the nucleated fragment. The delay in the nucleated fragment is associated with the polar body formation. It is likely, therefore, that the egg-nucleus, while it is active in forming polar bodies exerts a delaying action on the activity of the sperm-nucleus in forming the sperm-aster.

Digestion studies on salivary chromosomes. Daniel Mazia.

The relationship of protein and nucleic acid in the structure of salivary chromosomes of *Drosophila*, *Chironomus*, and *Sciara* has been investigated by the use of enzymes. Caspersson has shown that trypsin brings about disintegration of the achromatic regions. In the present work, nucleases were applied, and the history of the nucleic acid components was followed by means of Feulgen's reaction and by photography in the ultraviolet. Experiments with mixed nucleases demonstrated that the Feulgen staining material may be digested away without subsequent disintegration of the chromosome, which could still be demonstrated by the ninhydrin reaction. Photographs taken with light of wave-length 2650A indicated that the pyrimidine bases also were removed by this treatment. Comparison of individual nucleases indicated that nucleotidase (alkaline phosphatase of intestine), would alone produce the results described. This is interpreted to mean that the nucleic acid is attached to the protein of the chromosome through the phosphoric acid residues of nucleotides, as commonly assumed. These results contradict the theory of Wrinch. The problem of fibre formation by highly basic proteins has been attacked in the present investigation, and it has been found that thymus histone may easily be formed into fibres by compression of monomolecular films, although protamine does not form fibres when so treated.

Some properties of the residue from rapidly disintegrated Arbacia egg cytoplasm. M. J. Kopac.

Unfertilized *Arbacia* eggs were disintegrated in 0.53M KCl-solution at pH 6.8 to 7. Pigment and oil vacuoles, and granules were separated by centrifuging. Surface activities of the non-granular residue at oil-water interfaces were determined by: (1) measuring interfacial tensions with the flow-pressure method [Kopac, Colloquium on Micrurgy and Germ Free Technique, University of Notre Dame, in press] and (2) the estimation of interfacial adsorption by employing the drop-retraction technique [*Biol. Bull.*, **75**: 372, 1938].

The tension-lowering, Δ, is represented by the fraction, T_x/T_o, where T_o is the tension (dynes/cm.) at an *oil-water* [*0.53M KCl*] interface, and T_x is the tension at the *oil-water* [*0.53M KCl + residue*] interface. For cottonseed oil, $\Delta = 5.7/12.0 = 0.48$; for oleic acid, $\Delta = 0.6/7.5 = 0.08$. Similar values have been measured at *oil-protoplasm* interfaces using these oil phases and intact *Arbacia* egg cytoplasm.

The fraction of the total surface of an oil drop which is coated by surface-active molecules (proteins, protein complexes) within a given time is indicated by the adsorption ratio, δ. With cottonseed oil, δ equals 0.1 at 30 seconds and reaches a steady value of 0.2 in 10 minutes, while with oleic acid δ equals 0.9+ at 10 seconds and increases to 1 in less than 2 minutes.

The marked difference in tension-lowering of egg residue at cottonseed oil or oleic acid interfaces may be explained qualitatively by the difference in the amounts of proteins adsorbed by these oils. Accordingly, oleic acid showing the greatest adsorption also yields a lower interfacial tension when brought in contact with the residue.

By combining the values, Δ and δ, as obtained from flow-pressure and drop-retraction measurements, it is possible to relate the tension-lowering to adsorption. The following expression as developed from preliminary determinations agrees reasonably well with the data: $\Delta = T_x/T_o = n(1 - \delta)e^{-\delta}$. The correspondence between the observed and calculated tension-lowering at cottonseed oil interfaces is close ($n = 1$). However, with oleic acid the tension-lowering as calculated from δ is lower than the observed values ($n = 1+$ to 7). The values for δ are obtained from 10 to 30 seconds after the flow-pressure is recorded and one may therefore expect a greater adsorption at the time δ is measured particularly if the rate of adsorption is high. Also the elastic tension of adsorbed films of protein on oleic acid surfaces increases the flow-pressure so that the resulting interfacial tension values are higher than those expected on the basis of adsorption (from δ).

July 16

Ion intake by living cells. S. C. Brooks.

The present work is in marked contrast with the previously accepted conclusions as to the rate of movement of ions through the plasma membrane and the cytoplasm. These older conclusions were based on measurements of the total amount of ions in cells. Radioactive ions tell another story. When ions are transformed into heavier isotopes, e.g. Na_{12}^{24} instead of Na_{11}^{23}, they disintegrate and emit radiation, beta and gamma, which can be detected by very sensitive devices such as the Geiger-Müller counter. To obtain salts with activities high enough to be read and too low to injure cells, it is necessary to activate only one-billionth of the ions in the preparation. Under these conditions, it is considered that the concentration of the salt is essentially proportional to this radioactivity.

Cells are put into an excess of a dilute solution (0.0005M for Na_2HPO_4 to 0.033M for RbCl) in fresh or sea water or other normal habitat, according to the material. If the plasma membrane were rather impermeable to ions, it would be expected that active ions would be excluded. But these ions distribute themselves in a statistical equilibrium within an hour or two or in seconds, involving inorganic ion exchange. *Nitella* cells adjust themselves in about one minute for Na^+, K^+, Rb^+, and Br^-;

Spirogyra in less than 15 seconds; *Amoeba proteus* in less than 7 minutes, *Arbacia* eggs in 3–10 minutes for $HPO_4^{=}$ and Na^{+}, and other marine eggs and sperm, and a yeast were tried with essentially similar results. This means that these cells are very permeable to ions, the rates observed being about 10^{-7} to 10^{-4} G.M. cm.$^{-2}$ sec.$^{-1}$, in contrast with 10^{-9} to 10^{-8}, the earlier supposition.

Change in salt concentration of the immersion fluid produces results in accord with the ideas that: (1) equilibrium is attained with salts present free and ions occupying attachment points in protoplasmic constituents; (2) the entering ions replace all intracellular ions in proportion to their own concentration and the replaceability of the intracellular ions.

Fresh water cells, e.g. *Nitella*, do not easily give up active ions to distilled water, but do lose them in a few minutes to inactive salt solution. This seems to show that ions enter independently, cations in relation to acidic groups in the protoplasm and anions in relation to basic groups. These groups constitute an effective mosaic membrane, as suggested by earlier workers.

Later stages in ion intake are complicated with losses of salts, and primary accumulation. These are shown in cells sacrificed for each observation, and in cells kept intact through a series of observations. In the case of *Nitella*, the latter is possible since the sap does not participate in this ion exchange, thus showing low permeability of the vacuolar membrane. These losses of salts, thought of as loss of ion pairs, rather than by ion exchange, and primary accumulation, are connected with metabolism. This may mean that metabolically produced organic ions are normally exchanged for entering inorganic ions.

The use of radioactive tracers in the determination of irreciprocal permeability of biological membranes. L. I. Katzin.

The study of the work done by frog skin on the cations passing through it, exhibited in the phenomenon of "irreciprocal permeability," has been hampered by the low permeability of the skin, and the necessity of using similar solutions on both sides of the membrane. By the use of radioactive "tagged" ions (Na^{24}, K^{42}), it has been possible to make quantitative measurements over a range of mixtures of KCl and NaCl (total chloride always 0.12 N), using chemically similar solutions on both sides of the skin. It has been found that a marked irreciprocality exists for the passage of sodium ion, the rate inwards varying from 60 per cent (pure NaCl) to over 300 per cent (20 per cent NaCl/80 per cent KCl) greater than the rate in the opposite direction. This corresponds to an irreciprocality of about $12–20 \times 10^{-8}$ gram ions per hour per square centimeter of membrane surface.

It has been suggested by Steinbach (*J. Cell. Comp. Physiol.*, **10**: 51 (1937)) that an excretion of potassium chloride through the skin may occur. This is tentatively confirmed for the lower ranges of potassium concentration. The reservation is based upon an uncertainty in the interpretation of the amount of radioactive salt retained by the skin itself, which is of the order of magnitude to balance the irreciprocality value. The difference in rate of passage in the two directions across the skin is about $2–6 \times 10^{-8}$ gram ions per hour per square centimeter in the outwards direction.

Urethane and the respiration of yeast cells. Kenneth C. Fisher and J. Stern.

The inhibition of oxygen consumption in yeast cells by ethyl urethane has been examined. Since the inhibitor must operate by combining chemically or by adsorption with some portion of the cell, it seemed possible that the effect of different concentrations of the narcotic should be related to the concentration by an expression derived from the law of mass action. On plotting so as to test this hypothesis, the data imply that there are two respiratory systems which add together to make up the

normal oxygen consumption. These two systems become apparent when the preparation is treated with urethane because they are affected by different concentration ranges of this substance.

It is found that the concentration of urethane which just blocks cell division in these yeast cells closely corresponds to the concentration which just suffices to inhibit completely the oxygen-consuming system having the higher affinity for urethane. Similarly, the data of van Schommerberg on luminous bacteria suggest that in that organism also the respiration is composed of two fractions, the ability to produce light appearing to be associated with the system which is affected by low concentrations of urethane.

It thus seems that in these two cells the respiration is composed of two distinct fractions which can be characterized by their quantitative relations with urethane. Furthermore, there is a close parallel between the effect of the narcotic on the respiratory system which is more sensitive to inhibitor and its effect on cell division and light production.

Spectrophotometric determinations on hemoglobin and its derivatives. Matilda Moldenhauer Brooks.

Curves obtained by the spectrophotometer and the microphotometer with infrared Eastman plates at wave lengths from 11,000 to 6,000 mμ show again that methylene blue does not change the absorption maximum of fresh sheep blood. This indicates that no methemoglobin is produced. $NaNO_2$ produces methemoglobin, but the addition of KCN to methemoglobin in the concentrations and conditions used in clinical medicine does not change the absorption maximum.

The entire explanation of cyanide poisoning and its treatment is based upon the relative oxidation-reduction potentials produced in the respiratory enzyme (cytochrome oxidase). This enzyme contains a heme group with a reversible system, producing a shift in the $Fe^{++} \rightleftharpoons Fe^{+++}$. This reversibility is destroyed by cyanide not because the cyanide unites with the Fe^{+++} as is generally assumed, but rather because the KCN produces a low redox potential poising the system at this level so that most of the Fe remains in the bivalent form and can no longer be oxidized. The respiratory enzyme can only act at a definite positive potential and ceases to function when this potential becomes sufficiently negative and respiration stops. This appears to be the mechanism of inactivation by cyanide, by analogy with experiments on hemoglobin.

To produce recovery, it is only necessary to add a substance producing a positive potential. $NaNO_2$ or methemoglobin itself added will do this so that the Fe of the enzyme can again function at its proper potential. The *production of methemoglobin by $NaNO_2$ is a by-product and does not enter into the mechanism.* When methylene blue is used, not only is the potential poised at a higher level, but the dye can take the place of the respiratory enzyme by virtue of its catalytic property, as stated by the writer in 1932.

Finally a shift in the absorption band of hemoglobin has been reported by some investigators using a hand spectroscope, when KCN is added to methemoglobin in certain concentrations. In this case an absorption maximum at wave length 555 mμ appears. This is the absorption maximum of reduced hemoglobin and indicates that it is identical with it, rather than that it is a new substance known as "cyanmethemoglobin." This change fits in with the theory that the negative potential produced by adding sufficient KCN produces a valence change in the Fe of the heme radical from Fe^{+++} to Fe^{++} without necessarily combining with it.

JULY 23

An in vitro analysis of the organization of the eye-forming area in the early chick blastoderm. Nelson J. Spratt, Jr.

When a piece containing the entire eye-forming area in the ectoderm plus underlying mesoderm and endoderm (Clarke, 1936) is isolated from the chick blastoderm

at either the definitive primitive streak, head-process, head-fold, or early somite stages of development and cultivated upon the surface of a blood plasma clot *in vitro*. it forms, as a rule, a fore-brain with optic vesicles or cups of rather normal structure, The isolate is thus shown to have the capacity for developing a morphologically organized structure of a specific sort. The fact that isolates from the older blastoderms give this result more frequently than do comparable isolates from younger ones indicates a change in organization of the eye-specific area.

Isolates containing anterior and posterior parts, right and left halves, and fourths of the area produce, in general, corresponding parts of the fore-brain, e.g., either an anterior or a posterior portion, or a right or a left half. Such isolates from younger blastoderms show a greater tendency to regulate toward the whole fore-brain than do those from older blastoderms. The development of these isolates indicates a regional localization or specification within the area which becomes progressively more stable during development.

When a blastoderm from which a piece just large enough to contain the eye-specific area has been excised is explanted on the surface of a clot, the excised area is replaced by endodermal, mesodermal and ectodermal cells surrounding it. A node-like structure and primitive pit may then arise in the regenerated region (in some cases). Subsequently, medullary plate and neural folds form, and finally a complete fore-brain with eye vesicles develop in many cases. Regenerative capacity is greatest during primitive streak stages, markedly decreased in head-process stages, and apparently lost as somites begin to form. Since the regenerated region undergoes the same kind of morphogenesis that a normal eye-forming area undergoes, it is inferred that an eye-forming area has been reconstituted.

On the determination of the vascular pattern of the brain of the opossum. Ernst Scharrer.

In mammals there exist two types of cerebral vascular patterns: In the one, found so far in all Placentalia, the capillaries form an unending network; in the other, discovered by Wislocki and Campbell (1937) in the opossum, an artery and a vein are always associated in a pair and the capillaries do not anastomose but end in hairpin-like loops. The question to be studied concerns the factors that determine the type of vascular pattern. These factors can be sought in peculiarities of the chemical or physical constitution of the living brain (Wislocki, 1939), or they may be regarded as inherent in the cerebral vascular system. The influence exerted by the living brain on the angioblastic tissue was tested in experiments in which pieces of dead, formol-fixed brains from rats and guinea pigs whose brains are vascularized by networks, were implanted into living opossum's brain which is supplied by terminal arteries ending in capillary loops. After 3 to 4 months the dead brain tissue is invaded by bloodvessels regenerating from the surrounding living brain and the pia. The vessels penetrating rat's or guinea pig's brain are of the opossum type. Accordingly in the reverse experiment, when dead opossum's brain is implanted into living rat's or guinea pig's brain, no capillary loops are induced, but a network grows from the host's brain into the implanted dead tissue. From these observations it is concluded that under the conditions of regeneration the characteristic vascular pattern of the opossum's brain is not forced upon the angioblastic tissue by the peculiar chemical or structural constitution of the living nervous tissue of the opossum's brain, but appears to be determined by factors inherent in the vascular system.

Functional properties of transplanted and deranged parts of the amphibian nervous system. Paul Weiss.

Part of the experiments have been briefly reported in *Proc. Soc. f. Exp. Biol. and Med.*, vol. 44, p. 350, 1940. Additional data were obtained to show that structurally deranged nerve centers exhibit synchronized rhythmic endogenous activity. Even minced nerve centers upon recovery produce such activity. Transplanted reflex arcs

break down and give way to generalized activity. In all cases reflexes are of mass character and involve the whole isolated center without finer localization. It has been shown that the method of "deplantation" used in these experiments is potentially of the same value to physiology as tissue culture has been in the study of morphological problems.

July 30

A study of feather color patterns produced by grafting melanophores during embryonic development. B. H. Willier and Mary E. Rawles.

Melanoblasts (from neural crest) of various breeds or species of birds grafted into the right wing-bud of white and pigmented fowl embryos (host and donor each 70 hours or equivalent age) migrate into the host feather germs, producing an area of donor specific coloration or pattern in the down and later in the juvenile contour feathers of the wing and adjacent regions.

Melanophores from pigmented donors deposit melanin granules of specific size, shape and color into the epidermal cells of the feathers, which are structurally of host origin. Melanophores from white breeds likewise enter and occupy all available positions in the feather germs, thus excluding those of the host (pigmented breed) which come in later. Since these contain a lethal factor, they die before depositing melanin, with the result that the host feathers are white.

The color or color pattern produced in the host feather is specifically in accord with the genotypic constitution of the donor melanophore. Thus, barred-rock melanophores produce a barred pattern in non-barred host breeds (N.H. Red, White Leghorn, and Black Minorca). Melanophores from a female donor (1 gene for barring) produce a darker-colored host feather than those from a male donor (2 genes for barring). The sex of the host has no effect on the result. Similarly, melanophores from male and female embryos (sex of donor ascertained after hatching) of F_1 hybrids (R.I. Red ♂ × Barred Plymouth Rock ♀) showing sex-linked differences in plumage produce, respectively, barred and non-barred contour feathers in a white leghorn host irrespective of its sex.

Although the melanophore behaves to a large degree independently, its action is under the control of extrinsic factors. For example, the width of the black bar produced by barred melanophores in non-barred hosts varies with the rate of growth of the feather. Similarly, guinea melanophores produce in white leghorn feathers color patterns practically identical with those of corresponding guinea feathers. Thus the guinea melanophore in a particular feather produces a specific color pattern. Each feather germ apparently has certain physiological properties (rate of growth, threshold of reaction, etc.) peculiar to it, which controls the action of the melanophore in pattern formation.

The cellular basis of the color pattern in some Bermuda coral reef fish. (*Illustrated with color photomicrographs.*) H. B. Goodrich.

The observations presented were made in Bermuda during the summer of 1939. Four species of parrot fish and one wrasse were studied. The cell structures of the dermis overlying the scales were interpreted by means of a series of stereograms. In the dark-green parrot fish, *Sparisoma viride*, the dermis shows layers of chromatophores, iridocytes and next to the scale a thick stratum of loose connective tissue. Throughout the connective tissue are found intercellular blue pigment bodies. In *Sparisoma abildgaardi*, the red parrot fish, there are very abundant erythrophores in the belly region. There are also present numerous translucent intercellular bodies of unknown significance. Other parrot fish studied were *Sparisoma squalidum*, *Scarus vetula*, and *Scarus caeruleus*. The last two named species also showed an abundant blue pigment, in some cases diffusely distributed. The wrasse studied was

the bluehead, *Thalassoma bifasciatum*, in which the blue color is due to refraction of light. Slides were also shown of color-producing elements in *Holocentrus ascensionis* and in *Atherina harringtoniensis*. The paper was illustrated with about 50 kodachrome lantern slides, some taken by reflected light and others by transmitted light and at various magnifications including the use of the oil-immersion lens.

August 6

Production of a complex nitrogenous compound, related to tyrosine, by a species of Penicillium. A. E. Oxford.

Although the lower fungi show certain biochemical resemblances to the algae, especially with respect to their carbohydrate metabolism and in the production of the sugar alcohols, mannitol and erythritol, no peptides corresponding to those isolated by Haas and Hill (*Biochem. J.*, **25**: 1472 (1931); **27**: 1801 (1933); **32**: 2129 (1938)), from marine algae have so far been isolated from mold tissue. Since the latter contains dipeptidase and a variety of polypeptidases (see Johnson and Peterson, *J. Bact.*, **29**: 90 (1935)), the presence of appropriate substrates might reasonably be inferred. In the course of investigations on the carbohydrate metabolism of *Penicillium griseo-fulvum* (see Raistrick *et al.*, *Biochem. J.*, **25**: 39 (1931); **27**: 628 (1933); **29**: 1102 (1935); **33**: 240 (1939)), a crystalline and weakly acidic compound, of empirical formula $C_{22}H_{28}O_5N_2$, and m.p. 172°, has been encountered, the structure of which appears to be derived from that of an acylated tyrosine. The medium on which the mold was grown contained glucose and sodium nitrate as sole sources of carbon and nitrogen respectively, and the yield of the above product was relatively considerable, accounting for 5–10 per cent of the nitrogen supplied as nitrate. A partial structural formula can be deduced from the following facts: acid hydrolysis yields a terpene-like hydrocarbon $C_{10}H_{16}$, together with NH_3, CO_2 (2 mols.), acetaldehyde, and the known base *p*-hydroxy-ω-aminoacetophenone. Alkaline hydrolysis of the metabolic product yields NH_3 (1 mol.), and a crystalline acid $C_{17}H_{22}O_3$, which is split by acid hydrolysis to yield the hydrocarbon $C_{10}H_{16}$ and *p*-hydroxybenzoic acid. The metabolic product appears, therefore, to contain a β-ketotyramine residue etherified with an alcohol $C_{10}H_{17}OH$, and linked probably through a peptide linkage to a residue yielding acetaldehyde on hydrolysis. The molecule probably contains an acid amide group also and the following structural formula is tentatively suggested:

$$C_{10}H_{17}\cdot O\cdot C_6H_4\cdot CO\cdot CH(CONH_2)\cdot NH\cdot CO\cdot C_2H_3O.$$

It is noteworthy that the mold in question yields a great variety of non-nitrogenous phenolic metabolic products in addition to the above, suggesting a possible connection between its carbohydrate and its nitrogen metabolism.

Studies on erythrocruorin (invertebrate hemoglobin). Kurt Salomon.

(This work will be published shortly in the *Jour. Biol. Chem.*)

Photochemical spectrum of the Pasteur enzyme. Kurt G. Stern, Joseph L. Melnick and Delafield DuBois.

(A preliminary communication concerning this work has appeared in *Science* **91**: 436, May, 1940.)

August 13

Effects of ultra-violet light on respiration of the luminous bacteria. A. C. Giese.

Luminous bacteria suspended in buffered glucose solution were irradiated in quartz Warburg vessels with a Sterilamp which emits most of its radiations at

λ2537A. The irradiated bacteria show only a very slight increase in respiration and luminescence immediately after irradiation. After a lapse of time the irradiated bacteria show a decline in respiration which is proportional to dosage and indicates that either the concentration of the nutrient or of the enzymes has been reduced. Experiments demonstrated that the radiations affect the bacteria directly, not by altering the nutrients. The decline in the respiration and the apparent decrease in the effective enzyme concentration is proportional to the dosage and after irradiation is stopped, this decrease does not continue, for bacteria irradiated in salt solutions to which glucose is added at intervals for as long as nine hours after irradiation show comparable respiratory rates following each addition of glucose.

Irradiated bacteria are similar to controls in that they respond to peptone to a comparable degree and are affected by urethane and cyanide in a similar manner, but they differ from the controls strikingly in their constructive activities, for their respiration declines much more rapidly, indicating their inability to replace components necessary for maintaining a given rate of respiration. Such bacteria are also unable to reproduce for colony formation may be prevented in most of the bacteria without altering the rate of oxygen consumption and dosages which reduce respiration injure the bacteria to such an extent that less than one in a thousand form colonies.

When extracts obtained from bacteria injured by ultraviolet radiations were added to suspensions of bacteria containing no nutrient, a marked increase in respiration occurred; when glucose was present, a much smaller increase was observed; when both glucose and peptone were present, and the respiration was probably near a maximum value, the extract had no effect. The extract thus appears to act as a nutrient, not as an accelerator.

Effects of ether upon the development of Drosophila melanogaster. Ivor Cornman.

Eggs of various ages were exposed for 20 minutes to an atmosphere ⅓ ether by volume. This dose is in excess of that used for anesthesia of the flies, and was chosen to give a clear-cut ether effect. In the series most extensively studied, the eggs were 5 to 20 minutes old at the beginning of etherization. The effects produced were of three types.

1: Cytological Abnormalities. The embryos showed abnormal spindles and fused or scattered chromosomes. Giant nuclei and multipolar spindles with polyploid numbers of chromosomes appeared, but no embryo was uniformly polyploid. The cytoplasm was also affected, becoming distributed in abnormal patterns. Complete disorganization showed in eggs which had not hatched, where undifferentiated masses of cells and non-cellular cytoplasm were found.

2: Developmental Abnormalities. Mortality, as judged by hatching, was 40.5 per cent as against the 8.3 per cent mortality of the controls. The mean hatching time was 21.29 ± .10 hours as against 19.79 ± .06 hours for the controls, a delay of 7.6 per cent. Mortality and rate of development were affected adversely in the larval and pupal periods as well.

3: Phenotypic Abnormalities. Deformation of the abdominal segments in a manner similar to the mutation *Abnormal abdomen* showed in 15 per cent of the emerged adults. The incidence of this phenocopy appears to be less when older eggs are etherized.

Ether, then, can produce visible abnormalities in the early embryo. These, or other changes, result in slower development or death in the embryonic, larval, and pupal periods, and produce abnormal adults.

Neurosecretory cells in cockroaches. Berta Scharrer.

Neurosecretory cells, i.e. cells which in addition to their nervous character show histological features of gland cells, are known in vertebrates as well as in inverte-

brates. Several species of cockroaches, as representatives of the insects, are suitable objects to demonstrate to what extent a nerve cell can assume the character of a gland cell. Different types of neuroglandular elements within one species suggest different phases of a secretory cycle. These stages are in principle similar to those observed in vertebrates. There is a stage when only fine fuchsinophile granules are scattered over the cytoplasm. The cytoplasmic inclusions appear to increase in size and number and may fill the cell to such an extent as to impart to it the character of a gland cell rather than that of a nerve cell. Such granules are also seen to extend from the cell along the axis cylinder. Finally there are cells giving the impression of an endstage in the cycle.

The morphological evidence of secretion in the central nervous system of insects is of particular interest in view of the physiological results obtained in recent years, which proved that the central nervous ganglia exert an endocrine control over the processes of molting (Hemiptera) and pupation (Lepidoptera). By means of transplantation experiments in *Rhodnius*, Wigglesworth recently succeeded in localizing the molting hormone in the dorsal half of the central mass of the brain, i.e. the very region where in the same species neurosecretory cells are found. There is good evidence to suggest, therefore, that glandlike nerve cells are actually the source of hormones which control insect development. This is the first case in which the morphological evidence for a neurosecretory activity can be corroborated by physiological data.

The mechanism of the glass electrode. G. Haugaard.

The glass electrode is of interest to the biologist for two principal reasons. Primarily, the glass electrode has become an important tool for the determination of pH. Secondly, experiments on the glass electrode itself have interest in relation to biological membrane phenomena.

Cremer, publishing the first paper on the glass electrode in 1906, was concerned only with this second aspect, namely its use as a model to elucidate certain bioelectric phenomena. The pH scale was unknown at that time.

The most satisfactory glass for the preparation of the glass electrode is that developed by MacInnes and Dole. Therefore this has been used in the present experiments. By electrolysis experiments it is shown that the sodium ion alone is responsible for the passage of electric current through the glass membrane. When a glass electrode membrane is prepared so that one surface has been soaked in water for a long time to establish an equilibrium, the other side never having been in contact with water, the reaction of the "fresh" surface with water may be studied uncomplicated by reverse effects. Under this condition there is a quantitative relation between the sodium-hydrogen exchange and the potential alteration of the system.

Experiments comparing the uptake of hydrogen ions and water by MacInnes and Dole glass powder show that the ratio of absorbed hydrogen ions to absorbed water is a constant, hence the absorbed hydrogen ions are solvated. In alcoholic solutions it could be shown that the hydrogen ions also carry alcohol.

On the basis of the above experiments, the following picture can be given of what happens when a fresh glass electrode comes in contact with an acid, neutral or weakly basic solution (i.e. within the range where the glass electrode acts only as a hydrogen electrode). At first the glass electrode will take up water and the sodium salt of the silicic acid will dissociate under the influence of this water. Hydrogen ion at the same time is absorbed. In other words, the sodium salt of the weak silicic acid is partially hydrolyzed at the surface, forming in the surface layer a skeleton of silicic acid. The solvated hydrogen ions react readily with the surface, which affords an easy entrance for the hydrogen ions into the glass. In the middle of the glass membrane there remains a layer of intact sodium salt. This theory is an extension of a theory developed by MacInnes and Belcher and also of an earlier theory by Horowitz.

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Developmental changes in apical meristems. W. Gordon Whaley.

Apical meristems of several angiosperms were studied developmentally with respect to their rôles as functional embryos. The meristems were found to change greatly during development, the character and extent of the alterations being correlated directly with physiological differences. In longitudinal section the shape of the apical meristem changes from an almost flat structure in the seed to a strongly arched one in the mature plant. Volume of the meristematic region increases during the period of exponential growth, then becomes constant. Cell number follows essentially the same curve. Cell volume decreases progressively because the rate of division exceeds the rate of growth. The nuclei decrease in volume at a rate about two-thirds as rapid as the cells. As a result, there is relatively little cytoplasm surrounding the nuclei in the meristematic cells of mature plants.

Within a species or race a direct correlation was found between the size of the meristem and the size of the determinate organs which it produced. This correlation suggests that organ size inheritance in plants may be referred to the inheritance of meristem size.

The inadequacy of both Hanstein's three histogen (dermatogen, periblem, and plerome) and Schmidt's tunica-corpus system of meristem tissue designation was pointed out. The former resulted from an attempt to designate a specific 'germ' layer at the meristem while the latter fails to take into consideration several readily demonstrable differences between the epidermal layer and those layers immediately beneath it in plants with more than one tunica layer.

The relation between the four-carbon acid respiratory system and the growth of oat seedlings. Harry G. Albaum and Barry Commoner.

It was the purpose of this work to investigate the relation between the four-carbon dicarboxylic acid respiratory system and the various effects of auxin on oat seedlings. The effects of adding auxin and fumarate (which increase the activity of this system) and iodoacetate (which poisons this system) to the growing plants were determined.

The growth of the coleoptile is stimulated by the auxin in the seedling itself. When plants (of the variety Fulghum) were grown in various concentrations of iodoacetate the coleoptile growth was inhibited, the highest concentrations (.00005 to .0001 M.) resulting in a final size of but 50 per cent normal. The addition of auxin, and to a greater extent, of fumarate, negated the iodoacetate poisoning.

In contrast to the coleoptile, the growth of oat roots is known to be inhibited by the presence of applied auxin (10 mg. per liter). In the presence of iodoacetate this inhibition was partially removed, and conversely the inhibition was greatly magnified in the presence of fumarate.

Root number, which like the coleoptile length gives a positive response to this concentration of auxin, behaved like the coleoptile toward iodoacetate and fumarate.

It has been suggested by Thimann that all organs show a similar response to auxin, the direction of the effect being a function of auxin concentration. Thus, low concentrations stimulate, higher concentrations give an optimum plateau, and even greater concentrations inhibit. The different effect of the same auxin concentration on different organs is accounted for by the displacement of each of these curves along the auxin concentration axis, and also by the intrinsic auxin present in the particular species or variety. By testing the effect of various concentrations of iodoacetate on these phenomena, we have been able to confirm and extend this interpretation. When iodoacetate and auxin concentration are plotted in opposite directions on the same abscissa, and effect on the ordinate, it is possible to produce the hypothetical curves relating effect to active auxin concentration.

Respiratory changes following stimulation in Nitella. R. K. Skow and L. R. Blinks.

The large and very slow electrical disturbance following stimulation in *Nitella* is known to have so many of the properties of the nerve impulse that it seemed desirable to follow the changes in respiration during and after its passage down the cell. Oxygen consumption was measured in Schmitt's modification of the Fenn respirometer, using a travelling microscope on a micrometer screw mounting to follow the movement of the kerosene index droplet. The resting respiration of the cell (0.015–0.02 $mm.^3$ O_2 per minute) was increased 50 per cent to 100 per cent during repeated electrical stimulation (once per minute). By careful attention to temperature control it was also found possible to measure the changes following a *single* stimulation. An increase of 20 per cent or 30 per cent in O_2 consumption followed for some 10 or 15 minutes after a single propagated action current, gradually returning to the resting rate. Much smaller increases followed action currents restricted to only part of the cell; there was no increase on repeated sub-threshold stimulations, nor any volume change on continued flow of much larger currents through a dead cell.

A frequent characteristic of the respiratory response was a temporary *decrease* of the rate of movement of the index drop for about 5 minutes following stimulation, before the increase appeared. This was not a temperature artifact, but could represent either a momentarily decreased respiration rate, or an R.Q. temporarily greater than unity (the extra volume of CO_2 being a little too slowly absorbed by the KOH).

Partially in order to clarify this effect, an independent method of following CO_2 production was employed, using a thin film of $Ba(OH)_2$ on a filter paper strip brought very close to the cell, in a closed vessel of small volume. The electrical resistance rise of this strip during precipitation of the barium carbonate was followed in a bridge circuit. A marked increase of CO_2 production followed *immediately* after a single stimulation with this method, indicating no temporary decrease of CO_2 production. Whether ammonia production is possibly involved in the initial counter movement in the volumeter is still to be answered.

Neither irritability nor its accompanying excess CO_2 production could be abolished within periods up to 24 hours in purified hydrogen.

The relation of potassium to the bioelectric effects of temperature and light in Valonia. L. R. Blinks.

Damon has demonstrated the influence of KCl concentration upon the bioelectric potential of *Valonia*, while Marsh has described certain large temperature effects. The author has attempted to correlate these. Repetition of temperature studies with a great many cells (in sea water) shows a curious concave curve, with a minimum P.D. between 20° and 25° C., a sharp rise on warming between 30° and 35°, and a slower, though regular rise on cooling to 15°. (Cooling to 8 or 10° C. again lowers the potential, sometimes with poor recovery.)

The magnitude of the potassium effect (P.D. change on halving, doubling or quadrupling the KCl content of sea water) closely parallels the potential value in normal sea water at these temperatures. It is smallest at 25°, with the typical cusp and later fall of P.D. described by Damon at room temperature; at 15° it becomes flat-topped and appreciably larger; while at 35° the cusp is followed by a rapid rise to higher values. These differences may be due to the speed with which KCl actually enters the protoplasm, abolishing the experimentally altered gradient across the outer surface, and finally reaching the vacuolar surface, according to Damon's scheme.

Cells were also exposed to temperature changes while continuously bathed with sea waters of different K content. The temperature effect was almost abolished in 0.006 M KCl or lower; was normal at 0.012 M; and became considerably enhanced

in 0.024 and 0.048 M KCl. Almost exactly the same results were found with the effects of light (ascribed by Marsh to oxidation-reduction potential changes in the protoplasm). In K-free sea water there is no light effect, or sometimes a reversed one; in 0.006 M a slight effect; in 0.012 M the normal one; while in 0.024 or 0.048 M considerably increased effects. Evidently potassium closely controls the magnitude of these effects, probably *via* its altered entrance and accumulation under metabolic or other influences.

General Scientific Meetings

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The hydrogen ion and the osmotic concentrations of the cytoplasm in Vorticella similis (Stokes) as indicated by observations on the food vacuoles. S. O. Mast and W. J. Bowen.

The food vacuoles are formed by inward pressure of the cilia in the gullet. They are spindle-shaped and formation requires 50± seconds. During this period much fluid passes out, resulting in marked concentration of the solid particles in suspension.

After leaving the gullet they become spherical, decrease greatly in size (2± minutes) and their contents become acid (pH 3±). Two minutes later they increase very rapidly and greatly in size and their contents become nearly neutral (pH 6.9±).

The changes in the H-ion concentration in the food vacuoles were ascertained by observations on ingested yeast cells which had been stained by boiling them in solutions containing respectively Congo red, brom thymol blue and brom phenol blue.

The acid in the vacuoles is produced in the wall of the gullet and the cilia in it and condensed in the vacuoles, owing to differential permeability of the surface membrane. It is neutralized by rapid entrance of cytoplasmic fluid which is approximately pH 6.9.

The ingested bacteria die 16 seconds after the vacuoles leave the gullet when the acidity is pH 5±. Death may be due to this acidity.

If the concentration of the culture fluid is increased by 0.025 M (0.625 atmospheres) or more the body decreases in size. The osmotic concentration of the culture fluid used equals 0.79 atmospheres. That of the cytoplasm therefore equals 1.385 atmospheres. This doubtless varies directly with the concentration of the culture fluid owing to adaptation.

The decrease in the size of the vacuoles varies inversely with the concentration of the fluid in them, *i.e.*, the culture fluid, but they still decrease in culture fluid which is so concentrated that the entire body decreases in size and the osmotic concentration in them is higher than that of the fluid in the cytoplasm. This is due to inward pressure of the stretched membrane at the surface of the vacuoles.

The reversibility of certain artificially induced changes in the permeability of the erythrocyte. M. H. Jacobs and W. D. Jones.

The senior author and his associates have previously reported the production of striking changes in the permeability of the erythrocyte by narcotics, heavy metals (copper and mercury), salicylates, and especially by pH changes. The earlier results were obtained chiefly by the use of the hemolysis method which, while convenient and quantitative, may be complicated by factors not related to permeability. Much of this work has therefore been repeated, using the photoelectric method of Parpart to record the volume changes of the cells that occur when a small amount of a penetrating substance such as glycerol is suddenly added to a stirred suspension of the cells in isotonic NaCl. The results obtained by this entirely independent method

confirm very satisfactorily the earlier conclusions. In addition, both methods give evidence of a rather high degree of reversibility of most of the changes previously described. The permeability of human erythrocytes to glycerol, after being greatly reduced by M/4 ethyl urethane, may be restored to its original value by removing them to a urethane-free solution; and these changes can be repeated at least two or three times in succession. The greatly decreased permeability of human erythrocytes to glycerol produced by 10^{-5} M $CuSO_4$ may likewise be reversed in the same manner, or more readily and rapidly by the addition to the copper-containing cell-suspension of a very small quantity of serum or of hemolyzed cells. At a concentration of 10^{-5} M, $HgCl_2$ produces similar reversible effects. The greatly reduced permeability of human erythrocytes to glycerol at pH 5.0 or 5.5 may also be almost instantaneously restored to its normal value by adding enough alkali to bring the pH of the solution to 7.0 or above. On the other hand, the enormous reduction of permeability to NH_4Cl produced by low concentrations of tannic acid has so far proved to be reversible only to an inconsiderable extent by the methods used.

Oxidase activity and respiration of cells and cell-fragments. E. J. Boell, R. Chambers, E. A. Glancy and K. G. Stern.

The rate of respiration and the ability to oxidize *p*-phenylene diamine of mature *Arbacia* eggs is greater than that of immature *Arbacia* eggs. When the mature eggs are fragmented by high-speed centrifuging, the addition of *p*-phenylene diamine to the light 'halves,' containing the female pronucleus produces a larger increase in the oxygen uptake than is the case with the heavy 'halves' containing the granules. The 'disintegrum' obtained by shaking eggs in a calcium-free medium shows oxidase activity but no respiration.

The rates of respiration of mature and immature *Asterias* eggs are of the same order of magnitude. *P*-phenylene diamine and methylene blue, when added to immature starfish eggs, produce approximately the same increase in the oxygen uptake, the effects of the two substances being additive in character.

An attempt was also made to study the enzymatic activity of the large nuclei of the salivary gland of *Chironomus* larvae. The whole gland shows a definite respiration which is somewhat increased by glucose, pyruvate or *p*-phenylene diamine. Treatment of the glands with dilute egg albumin, at low temperature, abolishes the respiration but does not appreciably affect the oxidase system. Nuclei, isolated by this maceration procedure, appear to possess oxidase activity.

Respiratory metabolism of mating types in Paramecium calkinsi. Edgar J. Boell and Lorande L. Woodruff.

Respiratory metabolism of two mating types of *Paramecium calkinsi* has been measured by means of the Cartesian diver technique in order to determine whether a physiological basis for the difference in mating behavior between the types can be discovered.

In animals in which the mating reaction consists of 'agglutination' with subsequent pairing, the average rate of oxygen consumption per animal per hour is 0.25 mμl for Type I and 0.28 mμl for Type II. This difference possibly does not represent a real type difference in metabolic rate since the average size of Type II animals is slightly greater than that of Type I.

The oxygen consumption of animals in which neither agglutination nor pairing occurs is much larger than when both of these phases are manifest. The average respiratory rates for Type I and Type II animals are then, respectively, 0.43 mμl and 0.48 mμl per animal per hour.

Occasionally, the mating reaction consists only of the agglutinative phase without subsequent pairing. In such cases, the individuals of one type show the low rate of oxygen consumption characteristic of *Paramecia* which will both agglutinate and pair,

whereas the individuals of the other type respire at the typically high rate of animals in which mating tendency is absent.

The results indicate that an inverse relationship exists between metabolic level and mating tendency and that agglutination without pairing will occur between animals in which one type possesses a high rate of metabolism while that of the other is low.

Squid ink, a study of its composition and enzymatic production. Eric G. Ball and Pauline A. Ramsdell.

Ink expressed from the ink-sac of *Loligo pealii* contained 38.9 per cent organic matter and 5.7 per cent inorganic matter. The black insoluble material of the ink could only be centrifuged down after the addition of 3 volumes of 95 per cent ethyl alcohol. Partial purification was effected by resuspension in water and reprecipitation with alcohol, this process being repeated twice. The precipitated ink was finally washed with 95 per cent alcohol and air dried. A yield of 26 grams of dry ink was obtained from 380 ink-sacs. Qualitative tests on this material showed Cu and N to be present, Fe and S to be absent. It contained about 6 per cent ash, an amount which was not appreciably diminished by prolonged dialysis.

The ink-sac was the only tissue of the squid which yielded extracts capable of catalyzing the air oxidation of dihydroxyphenyl-alanine. Active enzyme preparations were obtained by grinding the sac with sand and 0.1 M Na_2HPO_4, centrifuging, and precipitating the enzyme from the black supernatant by the addition of an equal volume of saturated ammonium sulfate. The precipitate collected by centrifuging was dissolved in water, a black insoluble material removed and the enzyme precipitated by stepwise fractionation with $(NH_4)_2SO_4$. The fraction precipitated between 35–40 per cent saturation showed the most activity. About 300 mg. of such material was obtained from 380 ink-sacs. The protein precipitate of this fraction yielded colorless solutions and catalyzed actively the air oxidation of dihydroxyphenyl-alanine and catechol but not tyrosine. Dialysis against water inactivated the enzyme. Activity was not restored by addition of a concentrate of the dialysate. The activity of the enzyme was completely inhibited by diethyldithiocarbamate at a concentration of 1.3×10^{-4} molar. Other less effective copper poisons listed in the order of their effectiveness were K dithiooxalate, K ethyl xanthate, 8 hydroxyquinoline, and K ferrocyanide. Cyanide also inhibited the enzyme. Analysis of the enzyme preparation showed it to contain 0.033 per cent copper. It is concluded that the ink-sac of the squid contains a copper protein compound capable of catalyzing the air oxidation of orthohydroxyquinones. Hemocyanin, the copper blood pigment of the animal, possessed no such catalytic activity.

Observations on the occurrence of simple ethereal sulphates in marine algae. A. E. Oxford.

The micro-volumetric method devised by Øllgaard (*Biochem. Z.*, **274**: 181 (1934)) for the determination of inorganic sulphate has been modified so that the SO_4 of polysaccharide sulphuric esters may be determined simultaneously. After acidification of the liquid containing both inorganic sulphate (not more than 0.25 mg. S as SO_4^{--} per cc.) and ethereal sulphates with an equal volume of 25 per cent trichloroacetic acid, polysaccharides are precipitated by addition of 4 volumes of acetone, and to the supernatant is added water and finally a concentrated solution of benzidine in acetone in order that the final concentration of acetone may be 71 per cent by volume. The polysaccharide precipitate is hydrolysed by boiling N. HCl and the liberated SO_4^{--} precipitated as benzidine sulphate as in Øllgaard's method. Similarly, the total SO_4 (inorganic and ethereal) in the original liquid is determined after hydrolysis.

Cold-water extracts of numerous freshly gathered marine algae have been

examined by the above method, and in a few instances, notably *Dasya pedicellata* (C. Agardh) C. Agardh among the Rhodophyceae and various species of *Ulva* among the Chlorophyceae, the presence of polysaccharide sulphuric esters as well as inorganic sulphate has been demonstrated. The polysaccharides cannot be of great complexity since, once redissolved in water, they cannot be reprecipitated by the addition of alcohol or acetone. One specimen of *Ulva Lactuca* Linnaeus v. *latissima* undoubtedly contained a still less complex ethereal sulphate which could not be precipitated at all by acetone. Such compounds are of interest in that they may be regarded as intermediates between the SO_4 ion and the very complex polysaccharide esters of sulphuric acid such as agar-agar and the polysaccharide from *Chondrus crispus*.

Blood-sugar and the problem of the pancreas in lampreys. E. J. W. Barrington.

Previous work by the present author and by others has suggested that the zymogen component of the vertebrate pancreas may be represented in the ammocoete larva by an accumulation of secretory cells of a special type in the intestinal epithelium at the anterior end of the mid-gut, this region having a tendency to grow out into blind caeca in both larvae and adults of certain species. It has been known for many years that during the life of the larva there are budded off from the same region of the epithelium groups of cells which form gland-like follicles lying in the submucosal connective tissue, and it has been suggested more than once, on purely histological grounds, that these might represent the islet tissue of higher forms; if this could be definitely established, the lampreys would seem to exhibit an interesting early stage in the formation of the pancreas. The hypothesis has been tested in preliminary experiments by destroying these follicles in the ammocoete larva by cautery, and comparing the blood-sugar of the operated animals with that of animals upon which a control operation had been carried out. In a group of eighteen of the former, the mean blood-sugar value (measured 48 hours after the operation) was found to be 110 mgm. per cent, while in a group of twenty controls the value was 35 mgm. per cent. The difference is statistically very highly significant, and while clearly not in itself a proof of the existence of an insular mechanism, it provides the first physiological support for that suggestion.

Fatty acid compounds in the unfertilized egg of Arbacia punctulata. Albert E. Navez and Arthur B. DuBois.

Using the technique described in a previous note[1] based on extraction with cyclohexane after removal of water by centrifugal packing and alcohol treatment, "crude oil" of *Arbacia* eggs was prepared and fractionated.

The attention was centered especially on the glycerids fraction, reserving for later (after preliminary examination) the study of the small quantities of sterols, phospholipids and free fatty acids present in the crude oil. In the present work the red pigment mentioned in the previous note was removed by adsorption on norite before any further proceeding, in view of the fact that it seems to act as an oxidation catalyst for the glycerids. These are built up from saturated fatty acids and from unsaturated ones and both types have been recovered by Farnsteiner's lead salts method. The unsaturated fatty acids have been separated in the form of their bromine derivates and also by Tsujimoto's lithium salt-acetone solubility method. The mono-, di-, tri- and tetraethylenic acids are present; the relative proportion of each one is not constant throughout the season and does not seem to vary according to any obvious or definite rule. The same can be said about the relative proportion of saturated acids to the unsaturated although as a rule the quantity of unsaturated acids appears larger at the beginning of the season than at the end of the summer. All these variations are decidedly larger than the experimental variation.

[1] A. E. Navez, *Biol. Bull.*, **77**: 323, 1939.

The glycerids extracted and the fatty acids separated show marked spontaneous oxidation. By measurement of the oxygen consumption of thin films it is possible to show that the oxidation proceeds at possibly three different rates. After a short induction period the oxygen fixation proceeds at a relatively high rate for a lapse of time, then slows down to a new level for a period then again slows up further for a long period.

It seems probable also from investigation of the ease with which the oil can be extracted that the fatty compounds can and do exist in the cytoplasm in three forms:

(1) The "granular oil" associated with the fatty granules dispersed in the cytoplasm, easily attacked even at room temperature, by such solvents as cyclohexane and removed by means of a short hot extraction.

(2) Some "oil" released from the cytoplasm or cytoplasmic structures from which it can be detached by centrifuging according to the method used by Harvey for stretching the egg without breaking it in two parts and allowing it to round itself up again. The actual amount of energy involved in the process of stretching seems small *a priori*, but it is large enough nevertheless to "split off" this portion. Perhaps one should keep in mind that the same procedure brings also differences in behavior of "stretched and rounded" eggs in other processes (NH_3 release, enzymes).

(3) After treatment of eggs with an ammonium salt according to Heilbrunn's observation, the oil cap of centrifuged eggs becomes increasingly larger as the ammonium treatment lengthens. After such a treatment with ammonium chloride, it is possible to extract by the method indicated here an additional quantity of the crude oil. To all appearances this oil existed in the cytoplasm as bound to proteins.

Work on the actual composition of the three portions of oil, now in progress, will be carried on in the future.

Distribution of plasmalogen in certain invertebrate forms. C. Giddings.

The method used for the determinations was similar to that suggested by Feulgen. The values obtained show an interesting distribution in various tissues of invertebrate forms, and represent milligrams plasmal per 100 grams fresh tissue. Excess moisture was removed from the tissues by gently pressing between filter papers. Thymonucleic acid, glucose, etc. do not interfere. Highest values were obtained from the gills of mollusks (260–320 mg. from *Modiolus* and *Busycon* gill), whereas, for example, the gills of *Cancer irroratus* showed only 23–44 mg. plasmal.

High values were also obtained from the gonad and kidney of mollusks, with lower values from liver tissue.

Muscular tissue in general was also relatively low in plasmal content (often less than 15 mg.). Low values were also obtained from typical sponges, as well as from *Bugula*, *Perophora*, *Pennaria*, *Amaroucium* and Protozoa.

Lipoids and their probable relation to melanophore activity. G. H. Parker.

The chief activators of the melanophores in the catfish *Ameiurus* are three: intermedin from the pituitary gland, acetylcholine from the dispersing autonomic nerves, and adrenaline from the concentrating autonomic nerves. Catfishes blanch under the influence of adrenaline and darken under that of intermedin and acetylcholine. Dark caudal bands are formed by the action of intermedin and acetylcholine. Such bands can be formed in hypophysectomized catfishes where acetylcholine is the only dispersing activator present and may persist two weeks. Acetylcholine which will be destroyed almost at once in the aqueous fluids of the catfish is soluble in oil and in this solvent will remain active in a catfish for days. Is the dispersed state of the melanophores in a dark caudal band due to acetylcholine stored temporarily in the fish's lipoids or to continued activity of the dispersing nerves whereby new acetylcholine is produced? When a cold-block is put on dispersing nerve-fibers and after fifteen minutes the fibers are cut ventral to the block, the area of the nerve

darkens from the cut to the block but not beyond. If after an interval of time the block is removed from the fish on which the test has been made and the fish is allowed to swim, the peripheral part of the nerve area will darken. This interval of time may be as long as six and a half hours and represents a period over which the nerve has been producing acetylcholine. How much longer this process might go on is unknown. Nor is it known whether in the days that may follow during which the caudal band exists, the band is kept up by new acetylcholine from the nerve or by old acetylcholine which may have been stored in the adjacent lipoids or by both.

Studies of the distribution of the autolytic mechanism. S. Belfer, B. Bailey, H. C. Bradley and H. Eder.

A survey covering a wide range of forms shows that all vertebrate tissues examined possess the same well-defined type of autolytic mechanism found in mammalian tissues. Cathepsin is the characteristic proteinase of the system, with an optimum in the neighborhood of pH 4. No digestion occurs at pH 2, and very little at 7.5. It appears to be as universally present in active metabolizing vertebrate tissue as is respiration itself, and is probably of fundamental importance in protein metabolism. It is believed to mediate the synthesis and cleavage of proteins in hypertrophy and growth, and in the protein mobilizations which occur in starvation, involution, atrophy and necrosis.

Invertebrate tissues present no such single autolytic pattern. There are many different enzyme mechanisms represented, just as there are various oxygen carriers in invertebrate bloods. In some tissues there is a generalized autolysis over a wide pH range. In some there is evidence of cathepsin. In still others there are enzymes of the pepsin and trypsin type, acting at pH 2 and at 7.5. Thus squid and *Pecten* muscle digest over a wide pH range, but to a very limited extent. A slight optimum appears at pH 4. It is unlikely that these animals can mobilize muscle proteins to tide them over a prolonged period of starvation as do the vertebrates.

In the pedal muscle and the albuminigerous gland of *Busycon*, there is no digestion at pH 4, a marked maximum at pH 2, and a second smaller one at 7.5. The hepato-pancreas of *Busycon* digests slowly between pH 7 and 5, rising sharply to a peak at pH 2. If cathepsin is present it is completely masked by the pepsin-like enzyme present. The hepato-pancreas of the lobster resembles vertebrate gland tissue and appears to contain cathepsin.

The effect of various cholinergic drugs on the radula protractor muscle of Busycon canaliculatum. Carl C. Smith.

The radula protractor muscle of *Busycon canaliculatum* is admirably suited to physiological and pharmacological study. Preparations suspended in sea water remain viable for 36 to 48 hours. It appears to be histologically unique, consisting, according to Dakin,[1] of a central sarcoplasm surrounded by contractile sarcostyles. By careful dissection one can obtain good nerve-muscle preparations.

The response to most drugs of the parasympathomimetic type is a typical contraction. In the case of acetylcholine, the minimal concentration is usually 1 : 5,000,000, and 1 : 10,000,000 after eserine 1 : 100,000 has been applied. Nicotine 1 : 165,000 causes a slight contraction, but this concentration has no visible effect on the normal contraction produced by a just effective addition of acetylcholine. The effects of acetylcholine (1 : 1,000,000) or carbamylcholine (1 : 500,000) are almost entirely antagonized by both novocaine and cocaine, though the same concentrations and novocaine and cocaine will, by themselves, produce no effect.

Curiously enough, potassium ions even up to a concentration of 0.3 per cent or 3 : 1,000, have no effect on the muscle; higher concentrations cause a slow prolonged

[1] Dakin, Wm. J., (1912) *Proc. Trans. Liverpool Biol. Soc.*, **26**: 253.

contraction quite distinct from that produced by the choline esters. The relative insensitivity to ergotamine and ergotoxine differentiates this tissue from typical vertebrate smooth muscle.

Choline esterase in nerve fibers. E. J. Boell and D. Nachmansohn.

Recent investigations suggested that choline esterase may be concentrated at or near the surface of nerve cells and that the increase of enzyme concentration to high values at synaptic regions may be connected with the increase of surface due to the "Endarborisation" of fibers at synapses.

Direct evidence has now been obtained for this assumption. By means of the Cartesian diver technique, the enzyme activity has been determined separately in the axoplasm and the sheath of the giant fiber of the squid. Practically the total enzyme activity is found in the sheath, whereas the amount of enzyme present in the axoplasm is negligible. The $Q_{CH.E.}$ (mg. acetylcholine split by 100 mg. fresh tissue in 60 min.) was, e.g., in one experiment 0.420 in the sheath and 0.027 in the axoplasm, that is 17 times higher in the sheath. This is a minimum value, since a large part of the sheath is connective tissue. The enzyme concentration may be actually much higher than the activity per unit of tissue weight indicates, because the enzyme may be localized in a small fraction of the remaining volume. The figures found for the activity of the total giant fiber are, as could be expected, intermediate between those for the sheath and those for the axoplasm.

These experiments indicate that a high concentration of the enzyme exists at or near the surface of the nerve cell. They support the suggestion made by one of us (D.N.) in conjunction with previous observations that the activity of nerve cells may be connected everywhere at or near their surface with the metabolism of acetylcholine and that the phenomenon may be only quantitatively more important at synapses owing to the great increase of the surface. This may explain the findings of Lorente de Nó that acetylcholine can be liberated from fibers as well as at synapses and is compatible with the conclusion of Gasser and Erlanger that conduction of nerve impulses along fibers and across synapses differs only quantitatively.

Vascular reactions to renin and angiotonin. Richard G. Abell and Irvine H. Page.

Arterioles, capillaries, and venules in transparent moat chambers in rabbits' ears were observed with the microscope following the intravenous injection of renin and angiotonin [Page and Helmer, *J. Exp. Med.*, **71**: 29–42 (1940)] and the effect of these substances upon the blood vessels and blood flow determined. Comparable studies were made with epinephrine.

The intravenous injection of 0.2 cc. of renin was followed by arteriolar contraction. At the time of greatest contraction, 2.8 minutes after the injection, the arteriole photographed had become 0.24 its original diameter. Contraction was not complete and did not interrupt the flow of blood in any of the vessels. The renin had no appreciable effect upon the diameters of the capillaries and venules.

The intravenous injection of 0.2 cc. of Ringer's solution was not followed by contraction of any of the vessels.

The intravenous injection of 1 cc. of angiotonin was followed by arteriolar contraction, which became greatest 2.5 minutes after the injection. At this time the arteriole photographed had decreased in size to 0.53 its original diameter. Contraction was not complete and the flow of blood was not interrupted in any of the vessels. The angiotonin had no appreciable effect upon the diameters of the capillaries and venules.

The intravenous injection of 0.05 mg. of epinephrine was followed by very extensive contraction of the arterioles. Contraction became greatest 4.3 minutes after the injection, at which time the lumen of the arteriole photographed had

decreased in size to 0.04 its original diameter. As a result of arteriolar contraction, the blood flow in the arterioles and capillaries stopped. Injection of epinephrine was followed also by contraction of the venules. At the time of greatest contraction, 7.9 minutes after the injection, the venule photographed became 0.33 its original diameter. The epinephrine had no effect upon the diameter of the capillaries.

Factors affecting the frequency of contraction of the heart of Venus mercenaria. Albert E. Navez, John D. Crawford and Dora Benedict.

In the note published in 1939[1] conditions governing the isotonic contraction of the isolated heart were studied and among them the aeration and the addition of 1 part of dextrose to 250,000 parts of sea water in the bathing fluid, the loading of the muscle and the temperature were recognized as being of primary importance. Under these conditions after an initial rise of the frequency curve from the moment of excision to a maximum, in about two hours, then dropping very slightly to a new level, this plateau level could be maintained constant to within less than 1 per cent for a period as long as 120 hours.

For the isometric contraction one can generally state that the same conditions prevail, although the frequency does not rise as high as under isotonic contraction and the period of constant frequency of contraction of the isolated heart is reduced to 15–20 hours. The maximum tension which can be set up by an average heart weighing 125 mg. (net weight) can reach under optimal conditions of temperature and circulation, 2600 mg.

The diagram tension-time is a curve rising (in 1.5 hrs.–2 hrs.) from a low value shortly after excision to a sharp maximum (where the tension reaches twice the initial value), then declines to an intermediate plateau value in 5 to 6 hours from excision time. The value is then maintained for 10–15 hours, followed by a period of gradual decline and ends with complete exhaustion of the heart.

The frequency-time curve is of the same order except for the much broader maximum and for the delay in reaching this maximum.

In order to determine energy exchanges, measurements of O_2 consumption during isotonic and isometric contractions have been made, the heart being under different loads and the load being applied at one end or both ends of the muscle serving as suspension points and the load being attached to the middle.

A very good correlation exists over a wide range of loads between oxygen consumption and loading. The maximum work obtainable during isometric contraction and the rate at which it is performed correlate very well with the values obtained for critical loading of the heart and the time relations found for isotonic contraction.

Determining factors in the regeneration of Hydractinia. Charles E. Wilde, Jr.

Hydractinia echinata, a polymorphic, unbranched, colonial hydroid, exhibits, under certain conditions, a change of polyp form on regeneration. In the control series of experiments, pieces of stalks of gonozooids were severed from the living substrate and cut below the sporosacs. In all cases, in regeneration, the pieces reconstituted the gonozooid form.

In series A individual gonozooids were isolated, having attached at the bases a small amount of the living substrate. Cuts were made in the same manner as in the controls. In 94 of 116 cases, the stumps regenerated as gastrozooids.

Series B was then run, in which the polyps were treated similarly to series A except that a slanting cut was made so that the regenerate incorporated a small portion of sporosac-bearing region. Fifty-five of 64 cases regenerated as gonozooids.

[1] A. E. Navez and J. D. Crawford, *Biol. Bull.*, **77**: 315, 1939.

Gastrozooids were then isolated in the same manner. Cuts were made below the tentacular ring. In all cases the regenerates developed as gastrozooids.

Small pieces of sporosac region of gonozooids, stained with Nile blue sulphate, were grafted into the most oral tissues of gastrozooids cut below the tentacles. In 18 out of 90 cases the graft influenced the regenerate so that a gonozooid was formed, or a gonozooid and a gastrozooid.

A control seems to be exerted by definite regions of the polyps in regeneration. There is a gonozooid-influencing region in the most oral third of the gonozooid. The horny covered living substrate is the gastrozooid-influencing region. There appears to be a balance between the two regions, that which is dominant being expressed in the final form of the regenerate. The gonozooid influence seems to be the more potent per unit mass. Evidence for similar but less well-defined systems in *Hydractinia* dactylozooids awaits more data.

Time of determination and dominance in tubularian reconstitution. Edgar Zwilling.

An attempt has been made to study the process of reconstitution in *Tubularia* in such a manner that information might be obtained concerning such questions as to when the new hydranth is determined, and when the inhibiting effect of this new hydranth is exerted on lower levels of the stem. Young summer colonies of the hydroid were employed in the experiments reported. By the application of ligatures or small glass capillaries to the cut distal surface (a procedure which inhibits reconstitution when done immediately following section) at intervals, it was found that if the cut surface is exposed to running sea water for from 10 to 15 hours before the ligature or the cap is applied, then the hydranth would continue to develop despite the presence of the inhibitors. The hydranth may be considered as being "determined" after this interval. In addition, it was found that the number of proximal ends which reconstituted in these 10 mm. stems decreased as the number of distal hydranths increased. Further, when similar 10 mm. stems were ligatured in the middle at intervals after sectioning, it was found that the inhibition (dominance effect) of the proximal end by the distal was exerted during the latter part of the process—after at least 10 hours had elapsed. This seems to coincide with the time when the distal hydranth is determined. (The inhibition is evinced as a decrease in the number and size of the hydranths, and an increase in the time for formation.) It was also noted that the initial period of reconstitution was more susceptible to an inhibiting concentration of KCN. Stems placed in this solution after 5 hours exposure to sea water were much more inhibited than those placed in the KCN after 15 or 20 hours.

A regeneration-inhibiting substance released by Tubularia tissue. S. Meryl Rose.

If a small colony of *Tubularia crocea* is placed in 250 cc. of sea water and air bubbled through the water continuously, the solution becomes an inhibitor to regenerating stems. The degree of inhibition of regeneration is proportional to the length of time the inhibiting solution was in contact with the living colony. Four-hour colony water delayed regeneration approximately two and one-half hours in freshly cut 10 mm. stems. Twelve-hour colony water completely inhibited regeneration in most stems. More than 600 stems were observed in this experiment. Living adult hydranths also inhibit regeneration of hydranths. Even regenerating stems inhibit each other. Ten stems in 50 cc. of sea water regenerated more rapidly than either 20 or 30 stems in the same volume of sea water. This experiment was conducted eleven times with the same result every time. The degree of inhibition with crowding varied from a one-hour to a five-hour delay in the 30-stem group when compared with the 10-stem group.

The possibility that colony water and crowding inhibited regeneration because of oxygen deprivation or carbon dioxide accumulation was eliminated when the experiments were repeated in flasks through which air bubbled vigorously. Both the colony water and crowding effects were observed.

The inhibitor is thermally unstable. Heating the solution from room temperature to 80° C. and immediately cooling it destroys almost all of the inhibiting effect of twelve-hour colony water.

All of the experiments cited above were conducted between temperatures of 20° and 22° C. The crowding effect is scarcely observable at 18°. Other experiments have shown that ligatured stems do not commence to regenerate at temperatures between 19° and 23°. At temperatures from 14°–16° stems commence to regenerate even while ligatured in standing sea water. The coupling of these facts leads to the working hypothesis that the substance responsible for the crowding effect is produced in the tissue and must be removed before regeneration can occur. It is considered probable that the release of this substance at a perisarc-free surface accounts for regeneration at that point.

The rôle of oxygen in regeneration of Tubularia. L. G. Barth.

Experiments in which the oxygen concentration was varied between 100 per cent and .3 per cent showed that the oxygen consumption varied from 100 per cent to 29 per cent and the rate of regeneration from 100 per cent to zero. Further analysis shows that the time for regeneration is not affected in the higher oxygen concentrations from 10 to 100 per cent but that the size of the regenerant varies. At lower oxygen tensions both size and time were affected.

Other experiments showed a two-fold effect of oxygen. Nitrogenated sea water inhibits regeneration if the stems are placed in it immediately after cutting. If, however, the stems are first treated with oxygenated sea water for varying lengths of time and then placed in nitrogenated water, they will regenerate, the rate of regeneration increasing as the time of exposure to oxygenated sea water increases. A short exposure of 2 hours to oxygen delays regeneration but with 5 hours or more exposure, the time for regeneration becomes fixed and only the size of the regenerant varies.

From the experiments it is concluded that the effect of oxygen is two-fold. First there is a 5-hour activation period during which the time for the process of regeneration is determined. Second, the continual application of oxygen allows more and more cells to become involved in the formation of a hydranth.

The growth of oat coleoptiles after seed exposure to different oxygen concentrations. Harry G. Albaum.

The growth of *Avena* coleoptiles may be modified after grains have been soaked for 20 hours in solutions of glucose and 3-indole acetic acid as well as in water at different temperatures (Albaum, Kaiser and Eichel, in press). All these agents in the concentrations used lowered or raised the oxygen content of the soaking fluids. Within the limits of the experiments, the following relations obtained: the lower the oxygen content, the slower the velocity of growth, the longer the growth period and the larger the final coleoptile length.

The present experiments were performed in order to ascertain whether the same kind of relationships obtained when the oxygen content of the soaking fluids was varied more widely with oxygen-nitrogen mixtures.

Grains were soaked for 20 hours in water through which oxygen-nitrogen mixtures containing 0, 2.5, 5, 10, 20, 50 and 100 per cent oxygen were bubbled. After the treatment the grains were planted in beakers in contact with filter paper and the growth of the coleoptiles was recorded at regular intervals. The reciprocal of the time required to attain half final length was used as an index of rate of growth.

In addition, measurements of oxygen consumption were made with a Warburg respirometer at the close of the soaking period.

As the oxygen concentration was increased between 2.5 and 20 per cent, final coleoptile length decreased. Correlated with this decrease, there was an increase in rate of growth. In the 50 per cent mixture, final length and rate of growth both decreased. Respiratory rate, on the other hand, increased with increasing oxygen concentration up to 50 per cent. At 100 per cent final coleoptile length was inhibited markedly (84 per cent) as compared to the 20 per cent mixture, although rate of respiration and rate of growth were inhibited only to a relatively small extent (17–19 per cent). The last observation is of particular interest since it represents another case where growth may be markedly inhibited without bringing about a proportional decrease in respiration.

Preliminary experiments aerating grains in water for 0, 4, 8, 12, and 24 hours showed the effective period for altering growth rate and final size to occur some time after 8 hours.

Results of transplantation of the pituitary anlage to the thyroid region in Amblystoma. W. Gardner Lynn.

It has recently been demonstrated by Etkin (1939) that, in the frog, transplantation of either the pituitary or thyroid anlage in such a way as to bring the two developing glands in close proximity to each other results in precocious activation of the thyroid. This activation is strikingly evidenced by metamorphic precocity as well as by the histological picture presented by the thyroid. The evidence indicates the existence of some previously unknown thyrotropic influence emanating from the early pituitary, which is of the nature of a field effect rather than a hormone.

The present experiments were originally conceived as an attempt to ascertain whether this effect is concerned with the rate of differentiation of the activated gland. A series of 150 operations was performed on *Amblystoma* at Harrison's Stage 29. In all cases the pituitary anlage of one animal was transplanted to the thyroid region of another of the same stage. Donor, host and control were raised in small Stender dishes, duplicate series being kept at 10° and 25° C. The animals were preserved at intervals ranging from 4 hours to 25 days after operation and some were kept as long as three months. Stages were thus available ranging from the origin of the thyroid gland from the pharynx to well-differentiated glands with actively secreting follicles. Study of the sectioned material reveals no significant difference in the degree of development or activity of the thyroids of the control and experimental animals at any stage studied. Since in nearly all cases the transplanted pituitaries were found very near the thyroid and in many cases were in actual contact with it, and since the results in *Rana* under such conditions are so unequivocal, the failure of *Amblystoma* to show the effect must indicate that the urodeles differ markedly from the anurans with respect to this feature.

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Oxygen consumption of Arbacia eggs following exposure to Roentgen radiation. T. C. Evans.

Irradiation of either sperm or ova does not suppress the rapid increase in oxygen consumption which accompanies fertilization. Preliminary data indicate a probable relationship between level of oxygen consumption rate and rate of development. The rate of oxygen consumption, after the initial stimulation, appears to be higher in the control lots than in those irradiated with dosages which reduce the cleavage rate.

Effects of roentgen radiation on the jelly of the Arbacia egg. I. Disintegration of the jelly. T. C. Evans.

The results of experiments conducted this summer indicate that Roentgen radiation (at 5900 r/m.) has a direct effect on the jelly which normally surrounds the egg.

The radiation causes the immediate disappearance of the jelly from around the egg. Eggs placed in irradiated sea water or in water from irradiated eggs retain the jelly. The jelly is affected even in buffered solutions. The presence of the jelly in a solution of egg-water can be demonstrated by staining with Janus green. An irradiation of 59,000 r or more results in a negative test for the presence of jelly in the egg water. The power of agglutinating spermatozoa is greatly reduced in the irradiated egg-water.

Effects of Roentgen radiation on the jelly of the Arbacia egg. II. Changes in pH of egg media. By Marshall E. Smith and T. C. Evans.

Irradiation of sea water with dosages of 59,000 r does not significantly alter the pH. *Arbacia* eggs in sea water or in an iso-osmotic NaCl-KCl mixture irradiated with the above dosage produced a small but definite shift of the pH of the solution towards the acid side (pH 7.4 to a pH of 7.2 by a dosage of 25,000 r). When eggs were irradiated in solutions buffered at pH 7.4 this value remained unchanged.

Irradiation on jelly alone in the NaCl-KCl solution (59,000 r) reduced the pH from 7.4 to 7.0. The pH of the jelly solution was not altered when irradiated in a medium buffered at pH 7.4 with 0.01 M. glycylglycine. The jelly was disintegrated, by sufficient radiation, in both buffered and unbuffered solutions.

When eggs were cytolyzed by shaking in distilled water the pH was lowered to 5.5–5.9 and was not significantly affected by radiation.

A pH of 4.9 or lower was found sufficient to remove the jelly without any irradiation. There was little loss of jelly when the solution had as high a pH as 10.0.

It is concluded that irradiation by disintegrating the jelly and injuring the egg results in a lowering of the pH of the egg water.

Delay in first cleavage of Arbacia eggs following Roentgen irradiation of zygotes. E. P. Little and T. C. Evans.

The delay in time of first cleavage produced by irradiating the zygote immediately after fertilization yields a dose-effect curve which approximates a logarithmic character (as has been reported by previous investigators).

The character of the dose-effect curve can apparently be altered by changing the conditions of the experiment. Such factors, which appear to alter the dose-effect relationship are: (1) time of irradiation after fertilization, (2) intensity (if extreme) of the radiation, and (3) developmental conditions during and after the irradiation.

Concerning sensitivity of cells to X-ray. Grace Townsend.

Calcium release or ionic imbalance theories do not adequately explain the "fixation" of injury by fertilization (Henshaw, 1939) or gradient pattern sensitivity of embryos (Hinrichs, 1926). These may find interpretation in the postulate that X-Rays affect SS : SH ratio in the cell as determining potential held "fixed" as a mechanism of development. Kammerling (1937) and others report SH freed by irradiation.

Polarity of organisms is believed determined in the egg; multipolar cleavage in *Arbacia* (X-ray effect, faulty polarity?) is followed by faulty polarity of embryos. Oxygen tension and H-ion concentration may determine polarity of *Fucus* eggs, and the same factors affect SS : SH ratio (Voegtlin, Green). Sperm path may determine

polarity, I found tested sperm of high SH content. Polarity was studied by Child largely by relative reduction capacity of regions which appears to be related to free SH groups (Lund, 1931, Green, 1933, Maluf, 1933).

I found $Na_2S_2O_4$, glutathione, methylene blue, and ascorbic acid modified polarity of *Arbacia* embryos in relation to reduction potentials. X-ray effects were simulated by appropriate concentrations of glutathione: (a) in *Nereis* eggs activation and abnormal elevation of membranes, (b) in *Arbacia* delayed cleavage, exaggerated fertilization cones, multipolar cleavage, modified polarity of embryos, and "fixation" of injury by fertilization. Added glutathione sensitized *Arbacia* eggs to X-ray (3,500–7,000 r) resulting in cleavage delay and distorted polarity (by appearance) greater than the sum of the effects of the separate agents, also interfered with recovery.

Tumor tissue has a high SH and SS content and is relatively sensitive to X-ray. I found pea seedlings, soaked seeds, growing tips, and *Nereis* eggs of much higher glutathione concentration than the respective materials of comparison found to be far less sensitive to X-ray (Henshaw, 1933; Bruno, 1939).

The above and other phenomena (latent effects, high sensitivity of nucleus, etc.) cannot be solely explained by inorganic theories but could be interpreted by the postulate suggested.

Laboratory ripening of Arbacia in winter. Grace Townsend.

Twelve dozen sea-urchins distributed in four aquaria in two experiments were ripened Oct. 20–Dec. 20 and Dec. 21–Jan. 23 respectively. The temperature was regulated to near 18° C.–19° C. with occasional fluctuation. Algae, clams, and hard-boiled hen's eggs were the principal foods given though other marine forms were acceptable. Artificial light was supplied in the first experiment, but it was found in the second experiment that ripening occurred in total darkness as well as with continuous illumination. After one month's ripening October 18, dredged sea-urchins had normal appearing gonads but the females were frequently with oöcytes. After two month's ripening all of ten females yielded normal appearing ripe eggs which stratified normally when centrifuged and with a cleavage time of 55–70 min. at 21°–22° C. Eggs from summer urchins left standing had a cleavage time of 75–120 min. In the first experiment the gonad centrifuged volume from six specimen samples increased from 6.5 cc. to 12.9 cc. or 16.8 cc. for two experimental tanks with light, heat, and food supplied while controls with temperature unregulated regressed from 6.5 cc. to 3.4 cc. Summer ripened urchins left standing regressed to a sample volume of 2.2 cc. In the second experiment in one month of ripening the six specimen gonad sample volumes increased from 6.2 cc. to 8.0 cc.–8.9 cc. with light and to 10.0–11.8 cc. in darkness.

Occasional starfish and sand-dollar specimens with fertilizable eggs were observed in late December. *Nereis virens* spawns in March or April. The possibility of a winter supply of marine eggs at the laboratory is suggested.

A note on determining the sex of Arbacia punctulata. Ethel Browne Harvey.

Inject a drop of sea water saturated with KCl into one genital pore by means of a very fine hypodermic needle (No. 27). The eggs or sperm will almost immediately begin to ooze out from this pore alone, and the sex can be determined by the color (red ♀, white ♂). The shedding is stopped at once by placing the animal in a jar of still sea water.

Centrifugal speed and the Arbacia egg. Ethel Browne Harvey.

The size of the two "halves" into which the *Arbacia punctulata* egg is broken by centrifugal force varies with the speed. With a low speed of the electric centrifuge

(4,000 × g), the white half is twice the volume of the red. With the highest speed of the electric centrifuge (10,000 × g), the white half is 1½ times the volume of the red. With a low speed of the air turbine (50,000 × g), the halves are of equal volume. With the highest speed of the air turbine (100,000 × g), the red half is five times the volume of the white.

There is a difference also in the packing of the granules. With low speeds, the granules are well packed before the egg breaks in two; with high speeds, the egg breaks before the granules are well packed.

If the large red half obtained with high speed is re-centrifuged (10,000 × g), it stratifies like the whole egg but there is no nucleus. These large halves obtained with the air turbine develop much better than those obtained with the electric centrifuge, both the fertilized and the parthenogenetic merogones.

Colored photographs of stratified Arbacia punctulata eggs stained with vital dyes. Ethel Browne Harvey.

The different layers of the egg stratified by centrifugal force are stained differentially with different vital dyes, nicely shown with Kodochrome films. The jelly surrounding the egg is stained with Janus green (purple) and toluidin blue (pinkish lavender). The oil and nucleus are not stained. The clear layer is slightly stained with Nile blue (blue), toluidin blue (purple), rhodamine (pink), neutral red (pinkish yellow), chrysoidin (yellow) and Bismark brown (yellow). The mitochondrial layer is stained differentially with Janus green (blue), methyl green (purple) and gentian violet (purple). Yolk and pigment are stained blue with methylene blue, brilliant cresyl blue and Nile blue; purple with toluidin blue and methyl violet; red with rhodamine and neutral red; yellow or brown with chrysoidin and Bismark brown. With saffranin, the pigment is stained blood red, the yolk is unstained.

Elongation and return in spherical cells. Herbert Shapiro.[1]

When unfertilized *Arbacia punctulata* eggs are elongated into the dumbbell shape by centrifuging, and then transferred immediately to sea water, the course of return to normal spherical shape can be observed and measured microscopically. There is an initial rapid decrease in length, followed by a much slower, almost asymptotic approach to the spherical condition. Cells washed in calcium-free artificial sea water and then centrifuged, both elongate farther and round up more quickly than those kept in sea water or artificial sea water. Increasing the strength of calcium in the artificial sea water, while holding pH and osmotic pressure constant, results in cells rounding up more slowly than in normal sea water. These effects may be due either to alterations in surface forces, or the protoplasmic viscosity or both. According to current theory, the chief forces governing the division of a cell are its metabolism, surface conditions, and viscous properties. If one or more of these variables are altered as in these experiments, then the conditions imposed above should have an effect on the speed of cleavage. Unfertilized eggs washed thoroughly in sea water freed of calcium by the addition of a stoichiometric quantity of sodium oxalate and removal of the precipitate, go through first cleavage more rapidly than cells in sea water. This was observed by treating both sets of eggs identically, fertilizing them at the same time, agitating them gently in a water bath at constant temperature, and removing samples at five-minute intervals for fixation and subsequent counts of percentage cleaved. The complete curves of percentage cleavage against time after fertilization were then plotted. Calcium-free artificial sea water gave results similar to oxalated sea water. Artificial sea water with excess calcium was found, on the other hand, to retard cleavage, as compared with controls in normal artificial sea water.

[1] Aided in part by a grant from the Penrose fund of the American Philosophical Society.

Echinochrome as the sperm activating agent in egg water. Ivor Cornman.

It has long been known that eggs of various marine invertebrates produce substances which stimulate sperm, but the nature of the substance has never been determined. Recently Hartmann and others announced that echinochrome, the pigment which gives *Arbacia* eggs their reddish color, stimulates the activity of *Arbacia pustulosa* sperm. However, in experiments begun last summer at Dr. Eric Ball's suggestion, no stimulation of *A. punctulata* sperm by echinochrome could be detected.

Further experiments were carried out this summer under carefully controlled conditions. Most important is control of pH, since sperm are inactivated by weak acids and reactivated by bases. To eliminate any activation by pH difference, all media were held at the same pH by glycyl-glycine and piperazine buffers. The solutions of crystalline echinochrome were tested immediately as to their activating effect before any loss of the echinochrome by precipitation as a calcium salt, or by decomposition in the more alkaline solutions could take place. Several test series were carried out with suspensions at pH 6 for sea-water and pH 7 for isotonic NaCl solutions, in which the sperm were nearly motionless, but were readily activated by egg-water of the same pH. Under the same conditions echinochrome did not activate, in concentrations ranging from 1 : 25,000 (more deeply colored than egg-water) to 1 : 1,000,000,000. Other series carried out at higher pH values gave similar results. Clearly echinochrome is not the agent in *A. punctulata* egg-water which activates the sperm.

Tyler also reports no effect of echinochrome on *Strongylocentrotus* sperm activity or oxygen consumption. The discrepancy may be due to species difference, or to the fact that in the work with *A. pustulosa*, acidity was not controlled, since pH is not mentioned in any of the papers. By reason of its limited occurrence in invertebrate species and its inactivity in *A. punctulata*, echinochrome cannot be ranked as a general sperm-activating agent.

A relation between the dilution medium and the survival of spermatozoa of Arbacia punctulata. Teru Hayashi.

Spermatozoa suspended in seminal fluid (testicular plasma) were found to retain activity and fertilizing power longer than sperm in equal concentration in sea water. Microscopic observation showed that the sperm were more intensely active in the seminal fluid than in the sea water. Therefore the assumption that the death of sperm is caused by the exhaustion of an internal source of energy did not seem to be applicable to these experimental results. pH did not seem to be an effective factor in the survival of the sperm.

Saturation of the seminal fluid with ammonium sulfate brought down a precipitate which was filtered out. An artificial seminal fluid was made by dialyzing the residue against sea water to a volume approximating the original seminal fluid sample. The ammonium sulfate was removed from the filtrate in the same manner. This filtrate dialysate thus consisted of the original seminal fluid minus the precipitated constituent. Sperm suspended in equal concentrations in these preparations were found to live longer in the dialysate containing the precipitate. However, this artificial seminal fluid was not so effective as the natural product in keeping the sperm functional.

This precipitated constituent of seminal fluid seemed to be heat-sensitive. It is concluded that protein in the seminal fluid seems to be effective in prolonging the survival of sperm. The nature of the reaction between the sperm and protein is not known. There are three possibilities to be investigated; a nutritive relationship, a surface adsorption, and a relationship involving colloidal osmotic pressure.

The occurrence of cartilage at the bifurcation of the common carotid artery in an adult dog. William H. F. Addison.

In examining serial sections of the carotid sinus and adjoining regions in a series of fifty dogs, the presence of cartilage was observed on both the right and left sides in a large adult animal. The nodule of cartilage was about the same shape and size on both sides. It had a rounded form and measured nearly a millimeter in diameter. It was situated between the external and internal carotid arteries, as they arise from the common carotid artery. In this situation the walls of the adjoining blood vessels are highly elastic in structure and the nodule of cartilage is also traversed by elastic fibers. The nodule of cartilage, by reason of its size and position, causes slight bulges in the adjoining walls of the carotid sinus and of the external carotid artery. Cartilage is constantly found in a similar position in the young horse and is later replaced by bone. The bilateral position of the cartilage at the bifurcation of the common carotid artery suggests that the cartilage is a derivative of a branchial arch.

Cytoplasmic morphology in the gizzard of Gallus domesticus. Hope Hibbard.

Although general descriptions of the histological structure of the gizzard are available in the literature, the accounts of its development are incomplete. In the 1880's Cazin, and in 1925 Conselius, traced its embryology, but both studies lack cytological precision.

The observations in this report on the secreting epithelium of the chicken gizzard confirm the earlier accounts of an original simple epithelium which later becomes thicker, and at regular intervals, downgrowths into the dermis occur (not upgrowths of the connective tissue into the epithelium as described by Cazin). Subsequently, in each of these columns of cells a lumen appears through which the keratinoid secretion pours up to the surface. This is not a cornification of a stratified squamous epithelium as Kaupp (1918) has said.

The "Golgi apparatus," demonstrated by silver nitrate or osmic acid, figures prominently in these cells, changing from elongated parallel rods to a complicated network. Its interpretation in terms of aqueous vacuoles seems reasonable after neutral red postvital staining. A considerable amount of negative evidence as to its composition has been accumulated. It is not fat or lipoid (evidence from frozen sections); it is not glycogen (negative to iodine after absolute alcohol fixation); it is not mucus (negative to mucicarmine); it is not keratohyalin (negative to the Pasini stain). There are chondriosomes to be found in its neighborhood which sometimes compose a part of the blackened net.

A majority opinion exists that these glands do not secrete enzymes although Iliine (1913) claims the presence of a diastase. A mucoid secretion of certain cells of the gland, a secretion of keratohyalin by others, and the gradual disintegration and sloughing off of those cells nearest the gizzard lumen to form part of the lining, were observed.

Papers Read by Title

Further studies of photodynamic action in the eggs of Nereis limbata. Fred W. Alsup.

Parthenogenetic development can be induced in *Nereis* eggs by photodynamic action. The best development was obtained in eggs exposed in solutions containing 1 part rose Bengal and 200,000 parts sea water to light from a 1000-watt bulb. In these parthenogenetically developing eggs, first and second polar body formation and first cleavage show the same time sequence as in inseminated eggs developing normally in sea water. Later cleavages are usually irregular, but many of the

parthenogenetic eggs give rise to ciliated larvae which swim slowly and show varying degrees of differentiation.

Germinal vesicle breakdown due to photodynamic action can be prevented by the removal of calcium from the eggs. Eggs which were immersed in isotonic solutions of potassium or sodium citrate for 10 minutes and were then removed to solutions of rose Bengal in isotonic potassium or sodium chloride, did not show any germinal vesicle breakdown when subjected to photodynamic action. However, if eggs were removed from the chloride solutions to sea water containing rose Bengal, their germinal vesicles were broken down by photodynamic action.

When eggs were removed from sea water to isotonic citrate solutions containing small amounts of rose Bengal and were placed in the dark, some of them became heavily stained and showed germinal vesicle breakdown. The dye and citrates are believed to act in unison to produce a lytic effect on the eggs.

Although no photodynamic effects can be produced on *Nereis* eggs in the absence of free oxygen, the effects produced on eggs by concentrated solutions of rose Bengal acting alone in the dark[1] can be obtained in the absence of free oxygen.

The eggs of some females showed a small percentage (1–3 per cent) of germinal vesicle breakdown when they were exposed in sea water to artificial light.

A quantitative study of the effect of cyanide and azide on carbonic anhydrase. C. W. J. Armstrong and Kenneth C. Fisher.

The effect of the oxidative inhibitors cyanide and azide on living tissues exposed to them can be described by the mass law (Fisher et al., *J.C.C.P.*, vol. 16, p. 1, 103, 1940). The proportionality factor "a" in the mass law equation, which indicates the ratio of inhibitor to enzyme during the reversible inactivation, is found to have values characteristic of the inhibitor and system used. For cyanide four separate values are suggested averaging 0.4, 0.8, 1.2 and 1.6, while for azide "a" is 1. In attempting to elucidate the significance of the fractional values for cyanide, it is important to have similar data on any isolated enzyme systems which are sensitive to these inhibitors. Such data have now been obtained for carbonic anhydrase.

The enzyme preparation used was the crude chloroform extract described by Meldrum and Roughton (*J. Physiol.*, vol. 80, p. 113, 1933) and its activity was estimated by the "boat" method described by those authors. The values obtained are tabulated below:

	Cyanide	Azide	Azide
Initial pH	6.7	6.7	5.7
Final pH	7.6	7.6	7.0
"a"	1.07, 1.16, 0.91*	1.08, 1.01	0.91, 1.07

* Calculated from the data of Kiese and Hastings (*J. Biol. Chem.*, vol. 132, p. 281, 1940).

The average "a" of each set of data differs from unity by an amount which is within the limits of reproducibility of the experiments.

Upon the sources in the insect head of substances which influence crustacean chromatophores. Frank A. Brown, Jr. and Alison Meglitsch.

The American cockroach, *Periplaneta americana*, was used to determine the source of substances in the insect head possessing chromatophorotropic properties. Using

[1] See Alsup, *Biol. Bull.*, **77**: 324.

isolated pieces of integument of *Cambarus* bearing red and white chromatophores as the test material, it was found that the corpora cardiaca were the sources of a very powerful substance effecting concentration of crayfish red chromatophores. This tissue was still effective when extracted in more than 50,000,000 times its volume of salt solution. Qualitatively this gland was shown to differ from crustacean sinus gland in not possessing also a white pigment-dispersing action such as is found in the latter gland. Also, the rate of loss of effectiveness upon red pigment of dilution was shown to be much greater for corpora cardiaca than for sinus glands, suggesting a difference in the constitution of the red effecting principles of the two glands. Of the tissues tested, white pigment-concentrating material was found only in extracts of frontal ganglia and the brain while such material was absent from extracts of the thoracic ganglia. The corpora allata extracts possessed no chromatophorotropic activity.

Myofibrillar modifications in the caffeinized frog heart. Ralph H. Cheney.

The current studies present the microphysical modifications which appear in the striated myofibrillae of ventricular strips from *Rana pipiens* Schreber after immersion in caffeine. Two per centum caffeine-in-Ringer at pH 7.37 was used at room temperature for variable periods from three to thirty minutes. The material was fixed in Zenker's fluid and stained with phospho-tungstic acid-haematoxylin.

Normal controls and the caffeine experimental strips both showed a number of "spot disc" or local clot type areas as illustrated by Jordan (1912) and often involving only one or a few sarcomeres of one myofibril. An occasional transverse intercalated disc extending through a considerable number of myofibrillae of the muscle fiber was noted as described by Jordan (1912), who interpreted them as irreversible contraction bands. In addition, a variety of incipient or partial clot formations occur and seem to be identical with the microphysical changes due to congelation phenomena as induced by faradic current or chloroform in striated muscle by Nageotte (1937); by Speidel (1938 and 1939) by several types of stimulation in living skeletal muscle; and induced by caffeine immersion in the case of the frog gastrocnemius by Cheney (1939).

It is suggested that the sequence of physical changes in the myofibrillar shortening process is a constant in cardiac muscle as it is in skeletal muscle. However, a comparison of the degree of histo-physiological changes produced by caffeine upon striated cardiac tissue is never as drastic as similar concentrations for an equal time of immersion in the case of the striated skeletal muscle myofibrillae. Full retraction clots involving the complete disorganization of extensive myofibrillar areas with the formation of Zenker's wavy degeneration areas are absent. Even 2 per cent caffeine for a thirty-minute immersion period causes a degree of myofibrillar modification (injury) in frog cardiac muscle which is comparable to the effects of only a 0.03 per cent caffeine solution for an equal period in the instance of skeletal muscle. The cardiac myofibrillae are much less susceptible to caffeine action (injury threshold) than the striated muscle.

Effects of visible radiation on Arbacia eggs sensitized with rhodamine B. Leonard B. Clark.

Arbacia eggs sensitized by immersion in 1 : 25,000 solution of Rhodamine B in sea water and illuminated with light of 128,000 foot-candles before fertilization showed increase in time to secure 50 per cent cleavage, increase in number of eggs giving abnormal cleavage and increase in percentage of eggs undivided at the end of three hours after insemination and an increase in number of eggs cytolyzed with length of exposure.

By using the percentage of eggs remaining undivided three hours after fertiliza-

tion as a measure of the photic effect, the relation between effect and exposure yields a sigmoid curve with the inflexion point at 57 minutes exposure.

The mechanism by which light acts on the egg-dye complex is not clear, for eggs placed in radiated solutions of Rhodamine B show similar but less intense effects to eggs radiated in the dye. Eggs previously illuminated and placed in unradiated dye show no difference over controls placed in sea water.

Effects of cyanide on cleavage in eggs of Ilyanassa and Crepidula. A. C. Clement.

Uncleaved eggs of *Ilyanassa* were placed into solutions of KCN in sea water varying in strength from N/100 to N/20,000. In all of these cleavage progressed in apparently normal fashion for some time, except that in the stronger solutions, at least, the division rate was slower than in the controls. The observations were made on eggs which had been removed from their capsules and it should be mentioned that such eggs when kept in sea water did not survive beyond about 2 or 3 days and did not produce fully-formed larvae, although ciliated embryos were formed. In certain cases eggs in cyanide solution survived far longer than the controls, but it is doubtful whether in any of them differentiation progressed beyond the point reached by the controls. In N/100 KCN cleavage did not progress beyond about the 16-cell stage. In N/1000 KCN cleavage, though somewhat retarded, reached a fairly advanced stage, but then development appeared to cease even though the eggs survived for several days longer. In weaker cyanide solutions (N/5000, N/10,000, N/20,000) development progressed to the point of producing ciliated embryos, but eventually stopped.

Eggs of *Crepidula plana* gave a different result. Uncleaved eggs placed into N/1,000 or N/5,000 KCN were blocked but in most cases only after cleaving once or twice. On removal to sea water even after remaining in the original cyanide solution for as long as 3 days, further, though abnormal, cleavage occurred.

Thus the initial cleavage phase of development in *Ilyanassa* is rather insensitive to cyanide. This is in contrast to the situation in some other eggs—*Arbacia*, for example—where cleavage is readily blocked by cyanide. Conceivably, in *Ilyanassa* the energy for cleavage is supplied either through a cyanide-insensitive respiration, or through anaerobic processes. Evidence is not at hand to prove or disprove either of these conjectures.

The cell origin of the prototroch of Nereis limbata. D. P. Costello.

E. B. Wilson's study of the cell-lineage of *Nereis* (1892) established the fact that during normal development the prototroch originates from the four trochoblasts of the 16-cell stage, $1a^2$–$1d^2$ (Wilson's $a^{1.1}$–$d^{1.1}$). Each of the original trochoblasts, in two cleavages, gives rise to four cells, of which three remain in the prototroch, and the fourth lies above in the upper hemisphere of the larva. The twelve prototrochal cells put forth prototrochal cilia at about the tenth hour.

The study of the development of isolated blastomeres provides a means of verifying these observations and of ascertaining the capacity for self-differentiation of the isolated cells. After separation at the 2-cell stage, AB and CD continue to develop and each produces a partial prototroch at the proper time. When isolated at the 4-cell stage, A, B, C, and D, after continued cleavage, differentiate a ciliated prototrochal band. Isolating at the 8-cell stage, 1a, 1b, 1c, and 1d cleave and later differentiate prototrochal cilia, while 1A, 1B, 1C, and 1D do not become ciliated. When the blastomeres of the 16-cell stage are isolated, four cleave only twice and then differentiate prototrochal cilia. By progressive separation of blastomeres of a given egg at the first, second, third, and fourth cleavages it can be demonstrated that these four cells are the trochoblasts ($1a^2$–$1d^2$). In certain cases one may observe that three of the four division products of the trochoblast are ciliated. The apical cells

($1a^{1}$–$1d^{1}$) undergo more than two cleavages and may differentiate cilia of the apical tuft type. Neither second quartette micromeres (2a–2d) nor second generation macromeres (2A–2D) differentiate cilia. If an isolated trochoblast is permitted to cleave once, and the products separated, each product will later cleave but once before differentiating cilia. Such pairs of cells never cleave again, even though this ciliated unit may continue to swim about for two days or longer. In their capacity for self-differentiation the trochoblasts of *Nereis* therefore behave in essentially the same manner as those of *Patella*, described by Wilson (1904), and the isolation data are in agreement with the cell-lineage studies.

Blood clotting in Callinectes sapidus. James A. Donnellon.

The blood of the blue crab, *Callinectes sapidus*, contains the same cellular elements as those found in the crayfish, *i.e.*, amoebocytes, thigmocytes and explosive corpuscles.

Blood clotting involves both a cellular clot and a plasma clot. The plasma clotting takes place in two phases; first, a localized clotting around each explosive corpuscle, and secondly, a general clotting of the entire plasma.

Tissue extracts of the shell of the blue crab or of any of the internal structures bring about clotting both of the cells and of the plasma within a few minutes. Extracts of *Arbacia*, starfish, *Limulus*, mollusks and shrimp also hasten clotting. Isotonic solutions of various K salts and of $CaCl_2$, $BaCl_2$ and $SrCl_2$ produce the same effect.

Dilute solutions of fat solvents, hypertonic solutions, electrical stimulation, ultra-violet irradiation and mechanical stimulation also hasten clotting.

Isotonic $MgCl_2$, saturated $MgSO_4$ and a 10 per cent peptone solution will inhibit clotting indefinitely. Isotonic solutions of K-oxalate and Na citrate produce the same effect.

If the blood is oxalated and then filtered the cells can be completely removed from the plasma. If this cell-free plasma is then recalcified it will not clot. If cell extract is added to this plasma, however, clotting takes place within a few minutes. Extracts made of blood cells from *Arbacia*, starfish, *Limulus*, spider crab, *Venus mercenaria* and *Mactra* produce the same effect.

This effect of cell extracts on plasma clotting indicates that plasma clotting is initiated by a substance or substances liberated from the cells. In normal clotting several changes take place in the cells before the plasma clots; the granules in the amoebocytes break down, the thigmocytes cytolyze and the explosive corpuscles explode. These cell changes are initiated by the injury substance liberated when the animal is injured. Any substance which will preserve the cells will also prevent clotting, while tissue extracts and certain chemical and physical stimulants which breakdown the cells will cause clotting. Clotting also seems to be dependent upon the presence of Ca.

Effects of light and hormones upon the activity of young turtles, Chrysemys picta. Llewellyn Thomas Evans.

Between July 19 and August 18, 1940 each of five turtles was fed a total of 180 mg. of desiccated thyroid mixed with the food daily. Six turtles which received an average of 9 mg. of testosterone propionate [1] and seven untreated controls were fed identical food minus the thyroid. All 18 turtles weighed between 30 and 40 grams each.

Light-weight cages, suspended by light springs, to each of which were attached a stylus, recorded activity on 12-hour kymographs. Controls and thyroid-fed turtles were tested not only in normal daylight and dark but also in complete darkness for

[1] Kindly supplied by E. Oppenheimer, Ciba Pharmaceutical Products, Inc.

single periods of 24 hours. Those treated with testosterone propionate were tested only in normal daylight and dark.

By measuring only the periods of activity, but not the degree of intensity of activity, the following results, expressed in percentages, were obtained:

Under normal solar daylight conditions the controls were 20 per cent less active than those fed thyroid. In total darkness controls were 37 per cent less active than those fed thyroid. Controls were only 53 per cent as active in total darkness as in normal daylight, while turtles treated with thyroid were 81 per cent as active in total darkness as in normal daylight.

Those treated with testosterone propionate were 12 per cent more active, after treatment, under normal solar daylight.

Under normal conditions all turtles began activity soon after daylight and continued active until about noon; that was followed by relatively little activity, and after dark there was almost none at all. In total darkness, however, activity was sporadic; morning activity began at no fixed time and in a few instances the thyroid-fed animals were as active all night as in the morning. Controls, however, were quiet throughout the night and early morning hours. Activity of thyroid-fed animals was more intense during any given period of activity than that of controls.

Effects of testosterone propionate upon social dominance in young turtles, Chrysemys picta. Llewellyn Thomas Evans.

Where young turtles of different sizes were kept together, the largest dominated the group during feeding. Each of the others in turn dominated the next smaller. The dominating animal expressed its dominance toward another by climbing upon the carapace, snapping at or treading on the head, biting a foot, or approaching with open jaws. This behavior inhibited other animals so far as food-getting was concerned.

Groups were established as follows: I, II, III, IV, each with three animals of the same weight (within one gram); V and VI with three each, the largest being 12 to 17 per cent heavier than the smallest; VII to XVI with two each, the larger being between 25 per cent and 30 per cent heavier than the smaller. One turtle in each of I, II, III, IV and the smallest in V to XVI received, subcutaneously, testosterone propionate[1] dissolved in sesame oil. Dosage ranged from $3\frac{1}{2}$ mg. for a turtle of nine grams weight to 19 mg. for a 49-gram turtle. The others in each group received equal amounts of sesame oil only.

More than 450 observations were recorded during daily feedings in July and August, 1940. Scores, based on objective signs of dominance listed above, were kept for each animal.

The turtles treated with hormone in all groups were more aggressive than the controls (injected with sesame oil only), except in groups VI, VII and XIV in which the larger controls maintained their dominance in varying degrees throughout the period of experiment.

All turtles treated with hormone showed markedly enlarged and swollen cloacal region.

The use of urethane as an indicator of "activity" metabolism in the sea urchin egg. Kenneth C. Fisher and Richard J. Henry.

The effect of various concentrations of ethyl urethane on the oxygen uptake of fertilized and unfertilized sea-urchin eggs has been determined along with the effect of the same inhibitor on cell division in the fertilized eggs. When the quantitative relation between inhibitor concentration and effect produced is examined (Fisher and Stern, *Biol. Bull.*, 1940), there is found a discontinuity in the relation suggesting

[1] Kindly supplied by E. Oppenheimer, Ciba Pharmaceutical Products, Inc.

that two parallel systems are summated to give the normal oxygen uptake. It is found further that the concentration of urethane just sufficient to completely block cell division is very nearly the concentration by which the more sensitive of the two respiratory systems is eliminated.

In the unfertilized sea-urchin egg, on the other hand, the oxygen consumption is uniformly sensitive to urethane, that is, the evidence obtained from the effect of urethane suggests a single respiratory system. The quantitative characteristics of this system with respect to inhibition by urethane are similar to the same characteristics for the less sensitive of the two respiratory systems indicated in the fertilized egg. It appears that fertilization initiates a second respiratory system (c.f., Ballentine, *J.C.C.P.*, 1940) which is added to the system already functioning in the unfertilized egg, and it may be through this second system that the energy required for division comes.

The inflation mechanism of Spheroides maculatus. Mordecai L. Gabriel.

The peculiar inflating habits of many of the plectognath fishes have long been familiar, but no satisfactory account of the inflating mechanism has yet been published.

In the present work, specimens of *Spheroides maculatus* were studied by observations on the intact fishes and following surgical removal of various structures. The action of particular muscles was observed through celluloid windows sewed into the ventral body wall, and by inductorium stimulation in anaesthetized animals.

Contrary to Thilo's belief (*Anat. Anz.* XVI: pp. 73–87), the post-clavicular apparatus plays no part in the puffing act. The post-clavicles are quite motionless during inflation, and fishes with both post-clavicles and the associated muscles removed showed no impairment of the inflating power.

The anatomy of the puffing mechanism is substantially as reported by Rosén (*Ark. f. Zool. Stockholm*, Bd. 7; No. 30). The essential part of the apparatus consists of the powerful muscles of the first branchiostegal ray, which depress a pad covering the ceratohyals, thus expanding the mouth cavity and drawing in air (or water). The elevation of the ceratohyals forces the air into the air-sac, which is a ventral diverticulum of the stomach, partially separated from it by a sphincter-like ring. This "sphincter" is not very contractile and remains open throughout the act of puffing. It does not serve to retain air in the air-sac, and inflation takes place perfectly well after its excision.

Air is retained in the diverticulum by a strong oesophageal sphincter and by the pylorus. The flap-like breathing valve in the mouth does little or nothing in this connection, and fishes in which this was lacerated or removed inflated rapidly. The opercular valves prevent leakage during the compression stroke, but the distended state of the air-sac can be maintained even when they are held open or removed.

Micromanipulative studies on the nuclear matrix of Chironomus salivary glands. E. A. Glancy.

Microdissection studies on freshly dissected salivary glands in Ringer's solution indicate that the chromosomes and nucleolus are imbedded in a central jelly of a weak consistency surrounded by a material which is more fluid. This is in accord with Duryee's findings on germinal vesicles of ovarian oöcytes in amphibians and fishes. The presence of protein in both jelly and the peripheral fluid is inferred from the precipitation produced when saturated ammonium sulfate, acids and heavy metals are introduced into the nucleus by a micropipette. Proteins may be further demonstrated by employing the oil drop retraction technique (Kopac, *Biol. Bull.*, **75**: 372, 1938). The evidence for a jelly-like matrix is as follows: (1) the nucleolus

and chromosomes move as a unit when one of these is pulled with a microneedle, (2) the nucleolus and chromosomes persist as a compact mass when the membrane of an isolated nucleus is torn, (3) centrally injected oil drops remain in place, whereas the natural tendency of oil to rise occurs when peripheral injections are made. In the nuclear fluid naturally occurring spherical particles (Nebennucleoli of Bauer) show an average displacement of one micron per second (Brownian movement). The consistency of this fluid is close to that of 40 per cent glycerol in water. This is shown by the similar magnitude of displacement by Brownian movement of carbon particles (less than one micron in diameter) either injected into the nuclear fluid or suspended in a 40 per cent glycerol solution. These observations suggest that the consistency of the nuclear fluid is approximately four times that of water. It may prove significant that there is a structural similarity between the atypical salivary gland nuclei which never divide and soon degenerate, and the completely functional nuclei of oöcytes which are to undergo many cycles of division.

The reversible nature of the potassium loss from erythrocytes during storage of blood at 2–5° C. John Harris.

When human blood is stored aseptically at 2–5°, potassium diffuses from the erythrocytes into the supernatant fluid (DeGowin, Harris, and Plass, *J. Amer. Med. Ass'n.*, **114**: 855 (1940)). The rate of loss is essentially that of a first-order reaction. Equilibrium is reached in 20–25 days. Thus the factor responsible for the shift is immediately operative.

The suggestion that the ammonia formed during preservation causes the cells to become permeable to potassium was not substantiated by experiments in which ammonium chloride was added to blood.

In the following experiments human blood was stored aseptically at 5° and then placed at 25° or 37°. The erythrocytes of a blood-glucose mixture (see aforementioned paper) stored at 5° lose 30 per cent of their potassium in 5–7 days and are apparently freely permeable to this ion. When this blood was placed at 37° no potassium diffused from the cell for periods of 6 to 24 hours. A migration of this ion from the plasma to the cells was usually seen, the cellular content increasing about 5 per cent. Cells from a 5-day-old citrate blood mixture, when placed at 37°, increased in potassium content by 15 per cent in 4 hours, thereby regaining a considerable proportion of the potassium they had lost during storage at 5°. After 4 hours the migration was again in the direction of cell to plasma. Such an accumulation was also seen at 25° although to a lesser extent. This reversibility of potassium permeation was completely absent when sodium fluoride (.02 M) was present. Under such conditions erythrocytes from a 7-day-old blood-glucose mixture when placed at 37° lost potassium very rapidly, about 15 per cent during the first hour and 60 per cent in 5 hours. Likewise sodium fluoride (.05 M) was found to induce a rapid loss of potassium from fresh human erythrocytes at 25°.

Rabbit erythrocytes have shown somewhat similar results although an actual increase in cellular content has not been observed.

It appears that the potassium content of the erythrocytes of human and possibly rabbit blood is maintained in some way by the cellular metabolism. When cells are stored at refrigerator temperatures the metabolism is lowered and the potassium diffuses from the cell, establishing a new equilibrium. When these cells are then placed at 37° the original state tends to be regained. The particular function affecting this equilibrium is inhibited by fluoride.

Studies on the life history of Anisoporus manteri sp. nov. (Trematoda: Allocreadiidae). Arne V. Hunninen and Raymond M. Cable.

The adult stage of *Anisoporus manteri* sp. nov. occurs in a number of marine fishes including the northern pipefish, *Syngnathus fuscus*, summer flounder, *Para-*

lichthys dentatus, killifish, *Fundulus heteroclitus*, and four-spined stickleback, *Apeltes quadracus*. It differs from the two described species of *Anisoporus* in the size of the body, dimensions of the eggs (0.062–.068 by 0.035–.04 mm.), shape of the ovary, and number of papillae on the ventral sucker (three anterior, two posterior). The cercaria is a modified cotylomicrocercous form, developing in sausage-shaped sporocysts in the branchial region and digestive gland of *Mitrella lunata*. It has a double-pointed stylet which is 0.011 mm. long, and three pairs of cephalic glands with a single lateral and two median ducts on each side. The tail is peculiar in that it does not form a hollow, sucker-like cup, but contains a mass of glands whose ducts converge to form a protrusible papilla, by means of which the larva may become firmly attached. The main ascending excretory tubules are ciliated and extend to the mid-acetabular level where each receives an anterior and posterior collecting tubule. The excretory formula is $2[(2 + 2) + (2 + 2)]$; it remains unchanged during post-cercarial development. The cercariae penetrate and encyst in the hemocoele of marine amphipods, particularly *Carinogammarus mucronatus* and *Amphithoë longimana*. The metacercaria grows rapidly from less than 0.1 to over 0.7 mm. in cyst diameter. Adult structures become evident early in development and large metacercariae frequently contain eggs.

Histological studies on the problem of edema in haploid Triturus pyrrhogaster larvae. Cornelius T. Kaylor.

Edematous swelling of the body is the most common abnormality associated with haploid amphibian larvae obtained by experimental means. Occasionally it is also observed in diploid larvae which have developed from unoperated eggs.

In previous experiments on androgenesis in *Triturus pyrrhogaster*, 9 edematous haploid larvae and 1 edematous diploid control larva were preserved. The edema began in the living larvae at the time the circulatory system should have been functional. These larvae were sectioned and studied this summer. The edema was exhibited mostly in the head, pericardial and coelomic regions. The circulatory system of 8 haploid animals was not functional, as evidenced by the fact that blood cells were found only in the vitelline veins where blood cells are originally formed. Other vessels of the body were hypertrophied. In the ninth haploid larva only a few blood cells were present in the systemic circulation. The diploid larva also possessed a non-functional circulatory system. The heart was beating in all these larvae at the time of fixation.

The pronephros exhibited extreme hyperplasia: nephrostomes, tubules and collecting ducts were enlarged. The normal number of nephrostomes opened into the body cavity, the convoluted tubules connected with the collecting ducts and the latter were traced to an open cloaca. These observations suggest a functional compensation of the pronephric system for excessive amounts of body cavity fluids.

It would appear, then, that a non-functional circulatory system was responsible for the edematous condition of both haploid and diploid *I. pyrrhogaster* larvae.

Do carcinogenic compounds affect cell permeability? Balduin Lucké, Arthur K. Parpart and R. A. Ricca.

There are now available a number of chemical substances which on application to tissues induce cancer. The mechanism by which these substances affect cells is not known. However, since cancerous cells have been alleged to possess a degree of permeability different from that of normal cells, the possibility exists that a primary action of carcinogens upon cells is change of their surface properties and hence their permeability. If this be true for cells in general, it should be possible to detect this change in those cells in which permeability may accurately be meas-

ured. At present the only such cells are certain marine egg cells and mammalian erythrocytes.

In the present experiments the cells used were eggs of the sea urchin *Arbacia punctulata* and of the annelid *Chaetopterus pergamentaceus*, and erythrocytes of mouse and beef. The carcinogenic agents were choleic acid compounds of 20-methylcholanthrene, 1-2-5-6-dibenzanthracene, and 10-methylbenzanthracene. In addition, related non-carcinogenic compounds of phenanthrene, 1-2-benzanthracene, and acenapthene were tested.

The egg cells were exposed for a number of hours to saturated solutions of these substances in sea-water, after which their permeability to water was measured from curves of osmotic swelling in hypotonic solutions, using a diffraction method for following volume changes of cells (Lucké, B., Larrabee, M. G., and Hartline, H. K.: *J. Gen. Physiol.*, 1935, **19**: 1–17; Lucké, B., Hartline, H. K., and Ricca, R. A.: *J. Cell. & Comp. Physiol.*, 1939, **14**: 237–252). Unexposed cells were used as controls. The erythrocytes were shaken with water-saturated solutions of the compounds for 6 hours; their permeability was then determined for ethylene glycol, glycerol, erythritol, thiourea, the ammonium salts of acetic and butyric acids, and for chloride and sulfate ions. In addition, the lytic action of butyl alcohol on the cells was measured. In all cases, unexposed erythrocytes were likewise tested. A photo-electric method was used (Parpart, A. K.: *J. Cell. & Comp. Physiol.*, 1935, **7**: 153–162).

The results of these several determinations showed that neither the cells exposed to carcinogens nor those exposed to related non-carcinogenic compounds differed in their permeability from the control unexposed cells. This was true for both marine eggs and erythrocytes.

The development of the skull in the non-aquatic larva of the tree-toad Eleutherodactylus nubicola. W. Gardner Lynn.

The Jamaican tree-toad, *Eleutherodactylus nubicola*, is one of the few anurans which deposits its eggs on land and has no aquatic tadpole stage in its life-history. The animal never possesses the typical tadpole suctorial mouth and larval teeth, and neither external nor internal gills are every present. The development of the cranium and branchial apparatus is modified in relation to these features. A complete series of developmental stages has been sectioned and studied and wax reconstructions of the skulls of five critical stages have been made. Post-embryonic stages have been studied by the use of cleared specimens.

The chondrocranium differs markedly from that of *Rana* in the absence of the supra- and infrarostral cartilages and in the late appearance of the quadratocranial commissure connecting the quadrate with the trabecula. These structures are directly concerned with the support of the suctorial mouth in ordinary tadpoles. The synotic tectum and the tectum transversum are formed relatively late and closure of the foramen prooticum by growth of the taenia marginalis does not occur until after the orbital cartilages have enclosed the optic foramen.

The hyobranchial skeleton is represented in early stages by four paired cartilages connected to a broad central plate. Its later development is a direct transformation into the definitive, typical frog hyoid which is fully formed several days before hatching. The fourth ceratobranchial is lacking.

The osteocranium ossifies in a succession which differs from that found in *Rana*. The most noteworthy feature is the early appearance of the angular, squamosal and dentary which in *Rana* do not develop until the time of metamorphosis. Aside from these, most of the bones which are formed during or after metamorphosis in *Rana* are not present in *Eleutherodactylus* until some time after hatching even though, at hatching, this animal already possesses the adult body form.

The embryonic origin and development of the pharyngeal derivatives in Eleutherodactylus nubicola. W. Gardner Lynn.

Since the Jamaican tree-toad, *Eleutherodactylus*, possesses neither external nor internal gills and has no open gill-slits during its embryonic history, it affords an excellent opportunity for a detailed study of the origin of the various pharyngeal derivatives. Sections of a large series of embryos have been studied directly and by means of wax-plate reconstructions.

As in *Rana*, the first visceral pouch, the hyomandibular, is never well-defined but the rod of cells which represents the anlage of the middle ear cavity at late stages probably marks its position. Three branchial pouches are formed. The thymus body is derived exclusively from the dorsal wall of branchial pouch I and there is no evidence of the existence of the transitory thymus body which Maurer found as a derivative of the hyomandibular pouch in *Rana*. Branchial pouches II and III give rise to paired epithelioid bodies which originate from the ventral medial walls of the pouches and come into close relations with the systemic and pulmocutaneous trunks.

The postbranchial body is paired as in *Rana*, not unilateral as in *Amblystoma*. It is truly "postbranchial" in that it arises from the pharyngeal wall behind the most posterior visceral pouch.

The carotid glands appear quite late and, contrary to Maurer's findings for *Rana*, seem to be differentiated exclusively from the walls of the carotid arch.

A search for the "Kiemenreste" of Maurer has failed to reveal such structures and, in view of the absence of gills in this frog, this would seem to support Maurer's original conclusions as to their origin rather than Norris' later claim that they are of extra-branchial derivation.

The thyroid develops from the pharyngeal floor in the usual way and, as the author has shown in a previous study, becomes active very early; a fact which is doubtless correlated with the precocious course of bodily differentiation in this anuran.

Preliminary report on effect of indole acetic acid on growth of Chlamydomonas. Sister Maria Laurence Maher.

Reports on effect of auxin on growth of algae made by Brannon and Bartsch, Leonian and Lilly, Robertson Pratt and Yin indicate that different culture media may play a part in the different results obtained.

In these experiments pond water, glass-distilled water and soil solution were media used. These were made up with concentrations of $2\frac{1}{2}$-5-10-15-20-30-40 and 50 p.p.m. of indole acetic acid with each media alone and also with the media containing .1 per cent dextrose.

Ten-cc. amounts of solutions were placed in cotton-stoppered Pyrex tubes and inoculated with 1 cc. of solution containing cells that were previously taken from agar slants and placed in solution so that most of the cells were in a motile condition. pH was adjusted at the beginning of each experiment and when counts were made, to 7 by use of sodium hydroxide and acetic acid.

Rate of growth was determined by increase in number of cells shown by haemocytometer counts.

Higher concentrations in all cases showed definite stimulation. In pond water, the peak of growth was reached in seven days with a concentration of 15 p.p.m. of auxin giving best results.

In glass-distilled water, the peak of growth was reached in two weeks with concentrations of 30 and 40 p.p.m. giving typical growth curves. Further work on factors causing variation in different experiments is needed. Rate of change of pH and type of multiplication appear to play an important part in these variations.

Rapidity of growth in soil solutions makes counts difficult because of the tendency

of the rapidly growing cells to clump together. A number of cultures were rejected because of bacterial contamination and considerably more work with this culture is needed before conclusions may be drawn.

Further studies on the metabolism of cell fragments. Herbert Shapiro.[1]

From earlier work on the oxidative activity of the light and heavy fragments of the egg of the sea urchin *Arbacia punctulata*, obtained by centrifuging, the first measurements on their oxygen uptake, secured with Warburg manometers, were reported (Shapiro, H., *Jour. Cell. Comp. Physiol.*, **6**: 101, 1935). These experiments have been repeated and extended in order to study the effect of potassium cyanide on both unfertilized and fertilized "halves." Microrespirometers of the Fenn-Winterstein type were used, modified, after Schmitt, by the introduction of a bridge stopcock. Preliminary investigation shows that the light unfertilized halves are relatively little affected by KCN, whereas the light fertilized halves are markedly affected by this agent, their oxygen uptake being considerably reduced. The temperature at which the experiments are carried out may determine the inhibitory effectiveness. Cyanide also completely inhibits the cleavage of the fertilized light fragments. The unfertilized heavy fragments were cyanide-sensitive, and the oxygen uptake by fertilized heavy halves was likewise depressed, in most of the experiments, in the presence of cyanide.

Responses to acetylcholine and cholinesterase content of Cerebratulus. Carl C. Smith, Blanche Jackson, and C. Ladd Prosser.

Cholinergic systems have been indicated in the annelids (Bacq and Copée, *Arch. Int. Phys.*, **45**: 310, 1937) but in no lower groups of animals. Preparations of the dorsal longitudinal muscle of the flatworm, *Cerebratulus lacteus*, were suspended in sea water and the effect of acetylcholine examined. No response was obtained in a concentration of 10^{-4} acetylcholine hydrochloride without eserine. After eserinization, contractions were obtained in concentrations of 5×10^{-6} acetylcholine. Increase of the concentration of acetylcholine after eserine increased the rate and height of the contraction. This sensitization by eserine led us to examine the cholinesterase content by titration and manometric methods. Three manometric determinations gave an average CO_2 liberation of 214.5 cu.mm./30 min./50 mg. tissue, wet weight. This value was corrected for non-enzymatic hydrolysis. The esterase content of *Cerebratulus* is much higher than has been reported for other invertebrate body muscles; this esterase content may explain the high degree of sensitization to acetylcholine by eserine. This evidence is indicative of a cholinergic system in the flatworms.

Response of the heart of the compound ascidian, Perophora viridis, to pilocarpine, atropine and nicotine. A. J. Waterman.

Pilocarpine (1–2000 to 1–10,000) may cause immediate increase in the length of the abvisceral phases for 10–40 minutes after exposure is begun, but this increase is less than that provoked by mecholyl, acetylcholine and especially adrenalin. Increasing depressive effects soon ensue accompanied by great irregularity in number of beats. In contrast, the normal advisceral rhythm may be at first maintained but depression appears earlier than for the abvisceral phase and soon the advisceral count may be reduced to 1–4 in number. Except for these short interruptions, the abvisceral phase practically becomes continuous. Rate in both phases becomes

[1] Aided in part by a grant from the Penrose Fund of the American Philosophical Society. Part of the apparatus used was made available by the Beadle fund of Vassar College.

reduced and irregular, the interval between reversals is increased, and long pauses become increasingly common. One to 500 reduces heart action to spasmodic twitching at the ends in 12–15 minutes. There appears to be no hindrance to the immediate penetration of all drugs tested; small animals are less sensitive than large ones; the advisceral phase is always the first to show depressive effects; considerable variation in response is shown by different animals; and even inactive hearts recover in sea water.

Atropine acts as a depressant in proportion to the concentration. One to 100,000 is relatively non-depressive, while 1–50,000 stops most hearts, except for spasmodic twitching at the ends, after 4–7 hours. Preliminary exposure for 20–30 minutes to non- or slightly depressive concentrations, (1) inhibits the initial stimulatory action of pilocarpine although the subsequent depressive effects, which may be intensified, are frequently characteristic of pilocarpine, and (2) sensitizes the heart to depressive action of pilocarpine. Combining non-depressive concentrations of both drugs quickly provokes and intensifies depression and irregularity in both phases.

Nicotine similarly depresses heart action. One to 500,000 was without effect during one hour but later caused marked reduction, while 1–50,000 acted immediately.

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SOME EFFECTS OF TEMPERATURE ON THE FREQUENCY OF DIVISION AND ON THE VOLUME OF STARCH AND FAT IN CHILOMONAS PARAMECIUM

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Introduction

The present experiments were undertaken to further the knowledge of the metabolism of *Chilomonas*, using temperature as the variable factor, and noting its effects on the frequency of division and on the volume of starch and fat.

Chilomonas paramecium is a small biflagellated protozoan of elliptical shape, filled with numerous round particles. It has been known since the publication of Ehrenberg (1838), in which it was described, pictured and named. Its particles were noted but incorrectly described as food vacuoles. Since then many investigators have contributed to the knowledge of this organism. Schneider (1854) recognized the starchy nature of some of the particles. Fisch (1885) and Dangeard (1910) described chilomonads and added briefly to the knowledge of the organism.

Mast and Pace (1932*a*, 1932*b*, 1933, 1935, 1937, 1938) made two important contributions to the study of *Chilomonas*. First, they developed methods for cultivating the species in a bacteria-free solution of inorganic salts plus sodium acetate; second, they discovered that some of the particles in the chilomonads were composed of fat. In their experiments they observed the frequency of division and the volume of starch and of fat in various solutions at a constant temperature.

Chilomonas possesses four properties that make it admirably suited for physiological experiments. First, using the methods of Mast and Pace, it can be grown in sterile solutions containing relatively simple solutes. Second, it divides at a fairly uniform rate. Third, it lives and

[1] The author gratefully acknowledges the valuable suggestions of Dr. S. O. Mast and Dr. D. M. Pace, the patience of Mrs. L. Carper in obtaining equipment and supplies, and the many tolerant criticisms of Dr. M. E. Smith and others in preparing the manuscript, figures, and tables.

thrives in a temperature range extending over twenty degrees. Fourth, it possesses a fairly uniform number of particles of starch and fat which are undoubtedly forms of stored food material.

Materials and Methods

The organisms were obtained from a strain grown in this laboratory for many years and cultivated exclusively in bacteria-free solutions containing sodium acetate and inorganic salts. In recent years, the strain had been kept at temperatures between 24° and 27° C.

Table I

Culture solutions for *Chilomonas paramecium*. The acetate ammonium solution is that of Mast and Pace (1938); the acetate-free solution is a modification free of nutritive substances.

	Acetate ammonium solution	Acetate-free solution
Sodium acetate, $NaC_2H_3O_2.3H_2O$	248.8 mg.	—
Ammonium chloride, NH_4Cl	46.0	46.0 mg.
Dipotassium hydrogen phosphate, K_2HPO_4	20.0	20.0
Ammonium sulphate, $(NH_4)_2SO_4$	10.0	10.0
Magnesium chloride, $MgCl_2$	1.0	1.0
Calcium chloride, $CaCl_2$	1.16	1.16
Sodium chloride, $NaCl$	—	112.5
Sodium sulphate, $Na_2SO_4 \cdot 7H_2O$	—	47.0
Distilled water	100.0 ml.	100.0 ml.

The culture solutions used in the experiments are described in Table I. The acetate ammonium solution is that of Mast and Pace (1938) and was used in all experiments requiring a nutrient medium; the acetate-free solution was used in experiments in which no food was supplied to the chilomonads.

The water used in making all solutions was redistilled in a Pyrex still and condenser and stored in Pyrex flasks until used.

The chemicals were Kahlbaum's " für Analyses " or " highest purity " except the sodium chloride which was Merck's " for biological purposes." The calcium and magnesium chlorides were weighed out approximately and dissolved in water, the molarity of the solution ascertained by the Mohr (1856) method for determination of chlorides, and the solutions

were then diluted to the required concentration. The required amounts of the other salts were weighed out and dissolved in the required volume of water.

Sterility of all solutions was maintained by heating in a hot air oven at 85° C. for 30 minutes on each of three days or by heating in an autoclave at 15 pounds pressure for 15 minutes. Cultures contaminated with bacteria or mold appeared occasionally but were discarded immediately and replaced by uncontaminated cultures. The results presented in this paper were obtained with chilomonads growing in sterile solutions.

All new slides and glass tubing were thoroughly cleaned by boiling in a strong aqueous solution of soap, rinsing, immersing in sulphuric-chromic acid cleaning fluid, and rinsing thoroughly in tap and distilled water. The culture slides were made of Pyrex glass and each had two depressions; these were placed in clean square Petri dishes, two slides to each dish, and the assembly sterilized in a hot air oven at 150° C. for 60 minutes.

Two types of pipettes were used, the "capillary pipettes" for isolating individual chilomonads, and the "measuring pipettes" for measuring and transferring solutions. Both types were made as follows: Pyrex tubing was cut into appropriate lengths, one end of each length was flanged to hold a medicine-dropper bulb and stoppered with cotton. The other end was drawn to a capillary point or calibrated to measure 0.1 cc. of fluid. Five such pipettes were put into test tubes, which were stoppered and sterilized at 150° C. for one hour. The pipettes were rinsed and sterilized after each usage.

Eight temperatures, each constant to $\pm 0.25°$ C., were available in a large rectangular bath. A compartment at one end contained a refrigerating unit, another at the other end a heating unit, and both were controlled by Aminco Metastatic thermoregulators. Stirrers provided a uniform temperature gradient between the two end compartments. Additional temperatures constant to $\pm 0.5°$ C. were available in a small bath.

In all experiments on the frequency of division the "isolation culture" method was employed. In this method, one chilomonad was isolated in a depression; the frequency of division was calculated from the number of progeny produced in a given time. Isolation cultures were prepared as follows. The hydrogen ion concentration of the acetate ammonium solution, prepared and sterilized as described above, was adjusted to pH 6.8 with sodium hydroxide or hydrochloric acid. A small quantity (0.1 cc.) was put into the depressions of clean, sterile slides in Petri dishes. A few cubic centimeters of water were poured into the bottom, and the dishes were put into the bath at constant temperature. After the temperature of the solution had reached that of the bath, one

chilomonad was introduced to each depression. The dish was left undisturbed for about 24 hours, and then the number of chilomonads in each depression ascertained. One of these chilomonads from each depression was transferred to the corresponding depression of a new dish and the process repeated as often as desired. The number of chilomonads in eight depressions was determined in each observation.

From the counts of chilomonads obtained in this manner, the frequency of division in divisions per hour was calculated as follows. The mean number of chilomonads per depression was computed. The number in the geometric progression of 2 immediately below the mean was subtracted from the mean; the remainder was divided by the same number in the geometric progression of 2 to give the fraction of divisions more than the last complete division. The result was added to the number of complete divisions. An example will elucidate the method. If 59 chilomonads were found in 8 depressions, then each had an average of 7.4 chilomonads. The number in the geometric progression of 2 immediately below 7.4 is 4, representing 2 complete divisions. Thus, 4 was subtracted from 7.4, and the difference, 3.4, divided by 4 to give 0.85 divisions more than the last complete division. The total number of divisions was 2 plus 0.85, or 2.85 divisions.[2] Convenient tables were constructed to abbreviate the calculations. The final value was last divided by the number of hours between observations to give divisions per hour. The method of computing the number of divisions is given here in detail because it differs radically from the method used by Mast and Pace (1933, 1935) for determining the frequency of division of chilomonads in isolation cultures.

There was much variation in the frequency of division and in the volume of starch and of fat under identical conditions of nutrition and temperature, so statistical methods were used to ascertain significant differences. The methods are those described in standard textbooks of statistics, such as Gavett (1925).

[2] It must be pointed out that the method described here is actually only a method of first approximation. The exact number of divisions can be determined from any number of organisms by applying the following equation:

$$X = \frac{\log N}{\log 2},$$

where N is the average number of chilomonads per depression, and X is the number of divisions.

Experimental Results

The Effect of Temperature on the Frequency of Division of Chilomonas paramecium

Chilomonads were grown exclusively in isolation cultures at all temperatures available, and the frequency of division measured. The results of this experiment are presented in Fig. 1. The figure shows that the frequency of division rises from zero at 9.5° C. to a maximum at 26°–30° C., and then decreases to zero at 35° C. Below 30° C. the measured frequency of division reflects correctly the rate of reproduction because the organisms live and thrive indefinitely. Above this temperature, however, the actual frequency of division may or may not

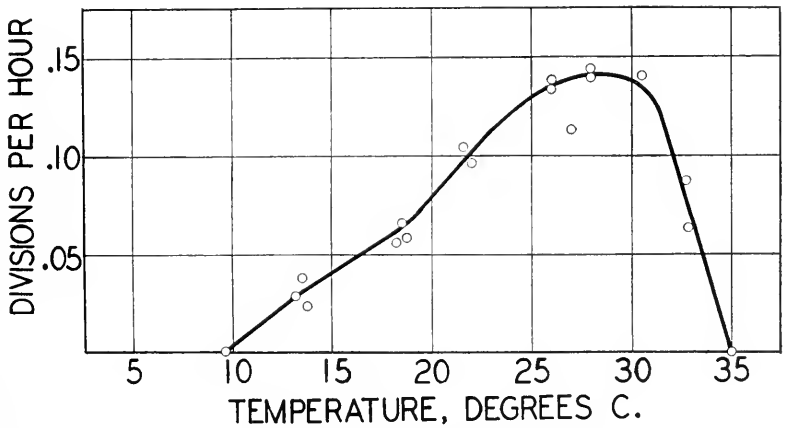

Fig. 1. The relation between temperature and the frequency of division of *Chilomonas paramecium.*

increase; the actual change is obscured by a decrease in the number of chilomonads brought about by death.

These experiments indicate that *Chilomonas,* like other living organisms, exhibits an increase in the metabolic activity (measured by the frequency of division) as the temperature rises to an optimum and then a decrease as the temperature rises still further. The reader is referred to Bělehrádek (1935) for an extensive review of this phenomenon.

An interesting observation was made in some preliminary experiments: for a short time after transfer of chilomonads from lower to higher temperatures, their frequency of division was low; gradually, however, it increased to that characteristic of the higher temperature. The experiment was repeated as follows to measure this phenomenon accurately and to seek other effects of low temperature on the frequency of division.

The organisms, apparatus, and materials were the same as in the preceding experiment. A clone derived from one chilomonad was grown at 27° C., and several from this clone were transferred to each of about 80 depressions and allowed to divide extensively at 27° C. These "stock cultures" were transferred to the compartment at 13.5° C. and left one week. Then isolation cultures were prepared and transferred to the six higher temperatures, where they were maintained and observed for two weeks. At the same time, the "stock cultures" were transferred to the compartment at 9.5° C. where they were kept for four weeks or until all chilomonads died. Isolation cultures were made from the "stock cultures" at intervals of one week, transferred to the seven higher temperatures, maintained and observed for a period of two weeks after establishment or, as was the case at 39° and 35° C., until all chilomonads died. The results are presented in detail in Figs. 2 and 3.

Figure 2 shows that during the first 48 hours after transfer from the low temperatures, the frequency of division increased from zero to that characteristic of the higher temperatures. Furthermore, after this period, the frequency of division remained constant at a value characteristic of the temperature studied, except that of those chilomonads exposed to 9.5° C. for 3 and 4 weeks, respectively, and observed at 30° C., which decreased to zero.

From this figure, three facts are evident. First, the exposure to low temperature modified the organisms so that their frequency of division was inhibited for a time after removal from the low temperature. Second, after the period of recovery from the effects of low temperature, the frequency of division became constant at a value characteristic of the temperature studied; in other words, exposure to low temperature had no permanent effect on the frequency of division. Third, all exposures to low temperature longer than one week had identical effects on the frequency of division at higher temperatures in which the chilomonads live indefinitely.

But what are the effects of exposure to low temperature on the frequency of division at temperatures in which the chilomonads do not live indefinitely? The following experiment answers this question.

Chilomonads were transferred from the "stock cultures" used in the preceding experiments to temperatures of 35° and 30° C. The chilomonads always die eventually at 35° independent of their treatment; at 30°, however, they normally live and flourish indefinitely. The results are presented in Fig. 3.

In the figure, the "percent surviving" was obtained by dividing the number of organisms living at the end of an observation period by the number introduced at the beginning and multiplying the result by 100.

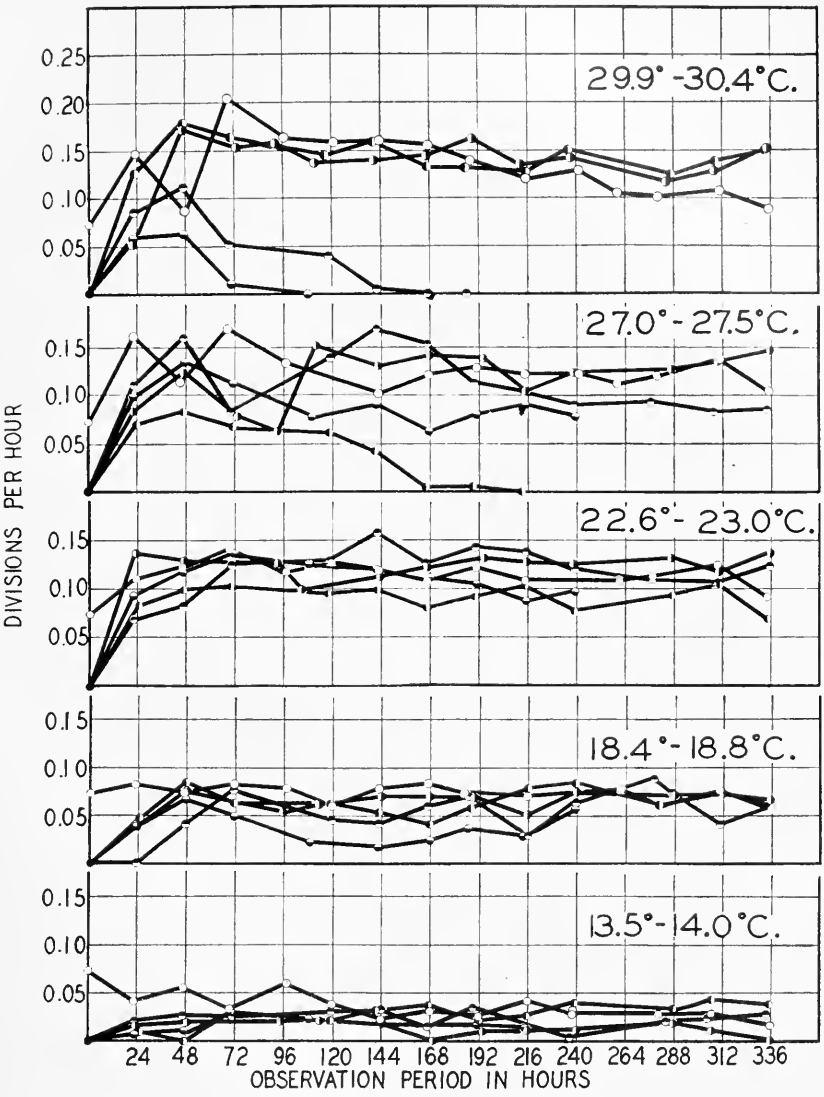

FIG. 2. The effects of prolonged exposures to low temperatures on the frequency of division at temperatures in which chilomonads live indefinitely.

○, exposed to 13.5° C. for 1 week.
◗, exposed to 13.5° C. for 1 week, then to 9.5° C. for 1 week.
◖, exposed to 13.5° C. for 1 week, then to 9.5° C. for 2 weeks.
◓, exposed to 13.5° C. for 1 week, then to 9.5° C. for 3 weeks.
◒, exposed to 13.5° C. for 1 week, then to 9.5° C. for 4 weeks.

Obviously, this "percent surviving" is the result of two opposing factors: the reproduction of the organisms on the one hand, and death of the organisms on the other hand.

Figure 3 shows that after the same period at 35° C., the percentage surviving decreased as the exposure to 9.5° C. increased. The figure also shows that after the various exposures to 9.5° C., the percentage surviving decreased as the exposure to 35° C. progressed. Furthermore, the figure shows that as the exposure to 9.5° C. increased, the time chilomonads lived at 35° C. decreased.

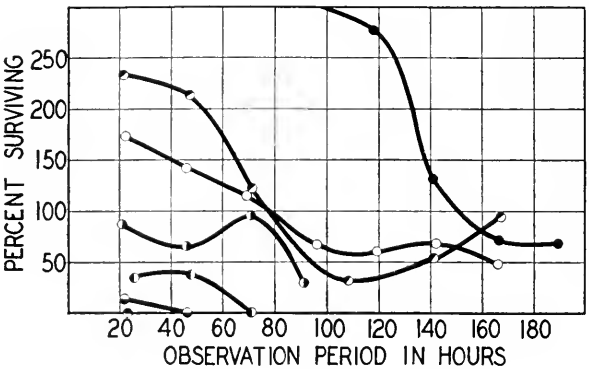

FIG. 3. The effects of prolonged exposures to low temperatures on the frequency of division at temperatures in which chilomonads do not live indefinitely.

○, exposed to 13.5° C. for 1 week, then observed at 35° C.

◑, exposed to 13.5° C. for 1 week, then to 9.5° C. for 1 week, then observed at 35° C.

◐, exposed to 13.5° C. for 1 week, then to 9.5° C. for 2 weeks, then observed at 35° C.

◓, exposed to 13.5° C. for 1 week, then to 9.5° C. for 3 weeks, then observed at 35° C.

◒, exposed to 13.5° C. for 1 week, then to 9.5° C. for 4 weeks, then observed at 35° C.

●, exposed to 13.5° C. for 1 week, then to 9.5° C. for 3 weeks, then observed at 30° C.

◍, exposed to 13.5° C. for 1 week, then to 9.5° C. for 4 weeks, then observed at 30° C.

It was noted above in Fig. 2 that the temperature of 30° C. acted as a viable temperature except when the chilomonads had been exposed to 9.5° C. for 3 and 4 weeks, respectively. Figure 3 also illustrates the effects of this low temperature on the percentage surviving at 30° C.: the same three phenomena evident at 35° C. appear. Experimental error accounts for the final rise in the percentage surviving of chilomonads subjected to 9.5° C. for 4 weeks.

Several conclusions may be drawn from these experiments. First,

exposure to low temperatures modifies the organisms in such a way that the frequency of division is low for a short time after transfer to higher temperatures. Second, the same modification apparently lessens the vitality of the organisms, because the ability to withstand detrimental effects of lethal high temperatures is reduced. Third, at temperatures in which the chilomonads live indefinitely the effects of the exposure to low temperature do not persist longer than 48 hours, but in other temperatures the organisms exhibit a decrease in survival that is in direct ratio with the length of exposure to low temperature.

Certainly the exposure to low temperature does modify the chilomonads. Modifications of this sort have been noted before. Dallinger (1887) induced modifications in certain monads that enabled them to live at very high temperatures and made it impossible for them to survive at the temperature in which unmodified monads flourished. Hance (1915) notes that a particular strain of *Paramecium caudatum* was able to resist higher temperatures than most and says that the former might have arisen in experiments dealing with high temperatures. Thus the present experiments serve to verify other observations, showing that organisms can be modified by environment and that the modification can persist for some time. The nature of the modification will be discussed again below under the effects of temperature on the changes in the volume of starch and fat.

The Effect of Temperature on the Volume of Starch and Fat in Chilomonas paramecium

The body of *Chilomonas paramecium* under conditions of optimum nutrition and temperature contains a fairly uniform number of particles; some of these give reactions characteristic of starch, some characteristic of fat. Under adverse conditions, the number and size of the particles may decrease or increase markedly. Mast and Pace (1932*a*, 1932*b*, 1933, 1935, 1937, 1938) make observations on the starch and fat of *Chilomonas* in solutions containing various substances always at one constant temperature. In their studies, they examined several chilomonads, then made a camera lucida drawing of a typical organism or of one taken at random. The present experiments were undertaken, first, to measure the actual volume of starch and fat in chilomonads, second, to observe the effects of different temperatures on the volume of starch and fat, and third, to ascertain the relation between the starch and fat and the frequency of division.

1. *In acetate ammonium solution.*—Experiments to ascertain the effects of various temperatures on the starch and fat under optimum conditions of nutrition were performed to supplement those of Mast

and Pace on starch and fat under various conditions at one constant temperature.

The organisms, solutions, chemicals, and glassware were precisely the same as in preceding experiments. The same methods were used to maintain sterility of the solutions, and the results appearing in this paper were obtained with solutions sterile except for the presence of the experimental chilomonads.

It was necessary to have a large number of organisms for each observation. To accomplish this several were transferred instead of the single chilomonad at establishment of each new culture.

The following procedure was used to measure the volume of starch and fat in *Chilomonas*. Several chilomonads from an experimental culture were drawn into a capillary pipette with the smallest volume of fluid; these were ejected on an ordinary glass slide, and surrounded by a ring of vaseline. Then one small drop of Lugol's solution and one of a saturated solution of Sudan III in absolute alcohol were added in succession with glass rods or wire loops. A cover-glass was placed over the drop and maneuvered to hold the drop in the center of the ring. This was observed with a microscope equipped with an ocular micrometer, and the size of each particle of starch and fat recorded. Five chilomonads were measured in each observation. Statistical methods were employed to ascertain significant differences.

Preliminary experiments showed that three temperature ranges could be distinguished: (*a*) the range between about 10°–13° C. and 30° C. in which the chilomonads live indefinitely (viable temperatures), (*b*) the range above the one in which the chilomonads live indefinitely (lethal high temperatures), and (*c*) the range below the one in which chilomonads live indefinitely (lethal low temperatures). Experiments were designed to show the effects of these three ranges on the volume of starch and fat.

a. Viable Temperatures.—The results of the experiments at viable temperatures are shown in the second and fourth columns of Table II. It is there shown that the volume of starch and fat per chilomonad at all viable temperatures is the same.

In order to obtain a more nearly complete analysis of the effects of viable temperature, the total volume of starch and fat synthesized by the progeny of one chilomonad in a 24-hour period was computed. This was done as follows. The number of divisions per 24 hours and thence the number of progeny produced from one chilomonad in that period were deduced from Fig. 1. By multiplying this number by the volume of starch and fat per chilomonad, the total volume of starch and fat synthesized in this period were obtained. The results are presented in

the third and fifth columns of Table II. It is there shown that as the temperature rises within the viable temperature range, the volume of starch and fat synthesized increases.

The remarkable part of these experiments is that although the rate of synthesis of starch and fat increases as the temperature rises within the viable temperature range, the volume of starch and fat *per individual* remains the same. One of two hypotheses may explain the situation. First, this may mean that under optimum conditions of salt concentration and nutrition in viable temperatures, the rate of synthesis of starch and fat controls the frequency of division. Second, the starch and fat may be by-products of metabolism, by-products which can be utilized as food materials under adverse conditions of nutrition, and their production and the frequency of division may take place at such rates that the volume of starch and fat remains constant.

TABLE II

The effect of viable temperatures on the volume of starch and fat per individual chilomonad and on the volume of starch and fat synthesized by the progeny of one chilomonad in a 24-hour period.

Temperature	Volume of starch per individual	Estimated volume of starch produced by progeny of one chilomonad in 24 hours	Volume of fat per individual	Estimated volume of fat produced by progeny of one chilomonad in 24 hours
30° C.	299.11±85.18	3806.87	52.07±20.12	701.67
27.3°	298.27±99.68	2907.53	66.92±22.48	652.08
23.2°	297.96±88.76	1348.15	65.99±32.76	298.43
18.5°	288.13±72.54	785.62	96.56±49.92	262.02
13.5°	314.18±61.15	518.86	119.50±34.01	197.34

b. Lethal High Temperatures.—The experiments were performed like those preceding, except that temperatures above the viable temperature range were used, that transfers were made at 24-hour intervals only when the experiment lasted several days, and that observations were made at 12-hour intervals. The results are presented in Fig. 4.

The figure shows that as the temperature rises above 32.5° C. the chilomonads lived for decreasing periods of time, and the volume of starch and fat increased as the exposure to the high temperature proceeded. This increase is due to two factors. First, the rate of synthesis either increased or was uninhibited. Second, there was a direct ratio between the length of life and the amount of accumulation. These two factors acted together to cause the greatest accumulation at 35° C.

It will be recalled that in experiments cited above, the frequency of division decreased as the temperature rose above the optimum; this, as

was pointed out, was largely due to the increase in the death rate and probably also to an actual decrease in the division. The present experiment demonstrates that the synthesis of starch and fat continues above 30° C., a temperature optimum for division, since starch and fat that would go into two or more dividing organisms now accumulate in one.

Thus the synthesis of starch and fat cannot control the frequency of division at lethal high temperatures. It is obvious that the frequency of division is inhibited whereas synthesis continues.

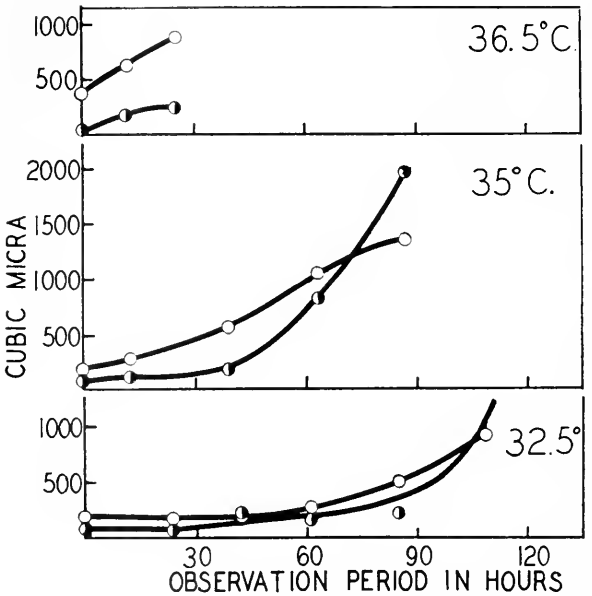

FIG. 4. The effect of three lethal high temperatures on the volume of starch and fat in *Chilomonas paramecium*.

○, volume of starch per chilomonad; ◑, volume of fat per chilomonad.

c. Lethal Low Temperatures.—These experiments were performed exactly as the preceding except as follows. A temperature of 9.5° C. was used since the organisms live for a considerable time but eventually die. Acetate ammonium solution was put into about 80 depressions, several chilomonads from a clone introduced to each, and the dishes put into the compartment at 27° C. for 24 hours until the chilomonads had become numerous. Then they were put into the compartment at 9.5° C. and observed periodically for 600 hours until the last chilomonad died. During this time the solution was replenished occasionally with fresh acetate ammonium solution. The results of this experiment are presented in Table III.

The table shows that as the period at 9.5° C. is prolonged, a decrease in the volume of starch is followed by an increase in the volume of fat. However, a statistical analysis shows that these changes are insignificant. The tendency for starch to decrease and fat to increase is, no doubt, present but is obscured by the great variation in the starch and fat in individuals under identical conditions.

It is quite probable that the modification of the frequency of division and the ability to survive at lethal high temperatures noted above as the result of prolonged exposure to low temperature is due to changes in the volume of starch and fat. However, experiments to be cited below indicate that the rate of resynthesis of starch and fat is so fast that the

TABLE III

The effect of a lethal low temperature, 9.6° C., on the volume of starch and fat per chilomonad.

Time at 9.6° C.	Volume of starch, cubic micra	Volume of fat, cubic micra
30 hours	165.4±35.9	35.9± 9.4
53	220.0±53.0	23.4± 14.0
90	138.8± 7.8	28.1± 3.1
124	37.4±25.0	57.7± 27.3
161	65.5±35.9	48.4± 39.0
211	28.1±25.0	129.5± 93.6
235	68.6±29.6	42.1± 18.7
292	51.5±10.9	84.2± 42.1
329	59.3±59.1	73.3± 25.0
353	106.1±26.5	73.3± 25.0
405	76.4±49.9	76.4± 31.2
497	71.8±54.6	173.2±138.8
521	99.8±64.0	248.0±126.4
598	64.0±28.3	224.6±163.8

modification can be accounted for only in part by changes in the starch and fat induced by low temperatures.

Since there is no division at this temperature and no increase in the volumes of starch and fat in the first 400 hours, there is no synthesis of these substances. Death could well be due to starvation in that no available food products are synthesized.

The decrease in the volume of starch followed by an increase in fat indicates that starch is probably transformed into fat.

2. *In Acetate-free Solution.*—Mast and Pace (1932*a*, 1932*b*, 1933) showed that if sodium acetate is omitted from solution, the volumes of starch and fat decrease to zero, and the chilomonads die of starvation. Their experiments were performed at one constant temperature, and they

made no actual numerical measurements of the volumes of starch and of fat. The present experiments were designed to ascertain the effects of temperature on the process of starvation of chilomonads in acetate-free solution and the effects of temperature on the recovery from starvation in acetate ammonium solution.

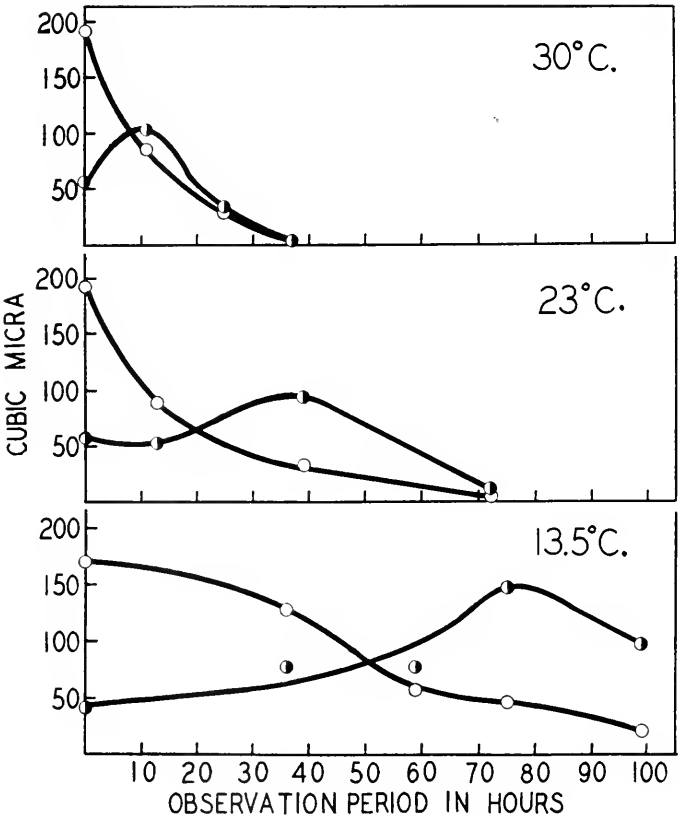

FIG. 5. The effect of viable temperatures on the change in the volume of starch and fat of chilomonads in acetate-free solution.

○, volume of starch per chilomonad; ◑, volume of fat per chilomonad.

Glassware and pipettes were the same as in the preceding experiments. The acetate-free solution described in Table I was used as the culture medium. The hydrogen ion concentration was adjusted to pH 6.8 with hydrochloric acid or potassium hydroxide, both prepared from boiled redistilled water. The Petri dishes were prepared as in the preceding experiments, except that a saturated solution of barium hydroxide

was poured into the bottom; this served to decrease the concentration of carbon dioxide in the atmosphere of the dish and to prevent the condensation of water beneath the depression slides.

A large number of chilomonads was transferred through four depressions containing acetate-free solution and then to the experimental acetate-free solution. It was then put into the constant temperature bath. No further transfers were made. The results of this experiment are presented in Fig. 5.

Figure 5 shows that the volume of starch decreased to a minimum, that the volume of fat first increased, then decreased to a minimum, and that these changes in the volumes of starch and of fat took place faster at higher than at lower temperatures.

Thus the same reaction occurs at the three temperatures studied; temperature acts as an agent that influenced the speed but not the processes involved in the reaction.

The changes noted here are similar to those indicated by the experiments cited above on the effects of lethal low temperatures. From these two sets of experiments, one can assume that under conditions in which there is no synthesis, the starch is gradually consumed, perhaps partially transformed into fat, and then the fat is consumed.

A second group of experiments was performed similar to the preceding, except that starved chilomonads obtained from cultures prepared as in the preceding experiment, were transferred directly to acetate ammonium solution. Synthesis of both starch and fat was rapid, requiring about 12 hours at 30°, about 15 hours at 23°, and about 18 hours at 13.5° C. for the volumes to increase from zero to the maximum typical of chilomonads grown in viable temperatures.

Discussion

The use of change in the volume of starch and fat as indicative of changes in metabolism of chilomonads raises three questions. First, is the method described in this paper sufficiently accurate to measure any changes in the volume of starch and fat? Second, is the change in the volume of starch and fat actually indicative of changed metabolism? Third, is the volume indicative of the mass of starch and fat?

The method is seldom very accurate. This is due to three factors: first, measurement of all particles is optically difficult; second, the particles are not always spherical, and their spherical diameter must be estimated; third, there is considerable variation in organisms kept under identical conditions. However, the method is undoubtedly more accurate than comparing organisms by means of camera lucida drawings or esti-

mating the relative amounts of starch and fat from a series of such drawings.

It is the author's contention that the volume of starch and fat may be indicative of the metabolic rate of the organism. The entire body of chilomonads living in acetate ammonium solutions, in natural ponds, or in rice cultures, is always well filled with starch and fat. This condition probably results from a balance between synthesis and decomposition of starch and fat; it is likely that in such an environment the chilomonads have a characteristic metabolic rate. A modification of the environment might upset this balance, causing a change in the rate of synthesis or decomposition, and subsequently, a change in the volume of starch and fat. Such changes would be accompanied by an alteration of the metabolic rate to a new and different characteristic value. Changes in the volume of starch and fat occurred and were described above; these were probably accompanied by changes in the metabolic rate.

The volume has been assumed in this paper to be an index of the mass of starch and fat. This is true if there are no changes in the chemical composition which alter the specific gravity of the starch and fat. Temperature is known to alter the iodine number of fats extracted from living organisms, but no experiments have been done on chilomonads to ascertain changes in the chemical composition of their protoplasm.

Summary

1. *Chilomonas paramecium* was grown in a solution of inorganic salts plus sodium acetate, and the effect of temperature on the frequency of division and on the volume of starch and fat measured.

2. As the temperature rises the frequency of division increases from zero at 9.5° C. to a maximum at 27°–30° C. and then decreases to zero.

3. When chilomonads are transferred from low temperatures to higher temperatures, a period of about 48 hours is required before the frequency of division increases to the value characteristic of the higher temperature.

4. When chilomonads are exposed to the low temperature of 9.5° C. for prolonged periods, then transferred to higher temperatures, the lethal high temperature and the period required to kill the organisms decrease. This indicates that the low temperature modifies the organisms and that the modified condition persists for some time.

5. At all viable temperatures, the volume of starch and fat per chilomonad remains constant, but as the temperature rises from 9.5° C., the volume of starch and fat synthesized increases, reaching a maximum at 30° C.

6. When organisms are exposed to lethal high temperatures there is an increase in the volume of both starch and fat. This is undoubtedly due to the fact that synthesis at these temperatures is not greatly impeded, that division decreases or ceases, but that the organisms live for a time and accumulate some starch and fat.

7. At lethal low temperatures, division ceases, there is a gradual decrease in the volume of starch accompanied by an insignificant increase in the volume of fat.

8. In solutions without sodium acetate, there is a decrease in the volume of starch to zero, an initial increase and a subsequent decrease in the volume of fat to zero. This process is identical at all viable temperatures but proceeds faster at the higher temperatures.

9. Synthesis of starch and fat by starved chilomonads in acetate ammonium solution is rapid and varies directly with the temperature.

10. The method of observing and measuring the volume of starch and fat has several drawbacks but does indicate changes with some accuracy.

11. The volume of starch and fat may indicate the metabolic rate of the organisms. Chilomonads living in an environment optimum for nutrition and temperature are filled with starch and fat and probably have a characteristic metabolic rate resulting from a balance in the rates of synthesis and decomposition of starch and fat. Changes in the environment may result in the breakdown of the balance, change in the volume of starch and fat, and probably a change in the metabolic rate.

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STUDIES ON EXPERIMENTAL HAPLOIDY IN SALAMANDER LARVAE

I. Experiments with Eggs of the Newt, Triturus pyrrhogaster

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Introduction

The problem of experimental haploidy has been investigated mainly in the Amphibia, since these eggs can quite easily be induced to begin development with the reduced number of chromosomes and the larvae are convenient material for microscopical studies.

In general, the results of many experiments using different methods and species have been that the development of the haploid embryos is abnormal. If development continues into larval stages, the animals usually, at one time or another, show certain symptoms of haploidy, namely, stunted growth, reduced activity, and an edematous swelling of the body. This is in direct contrast to the fact that natural haploids are known to exist in many species of arthropods and that viable experimental haploid plants have been produced. However, some haploid salamander larvae have been experimentally produced which were relatively free of some of the serious symptoms associated with haploidy (see review of the literature by Fankhauser, 1937). The most striking example was a metamorphosed *Triton* larva obtained by Baltzer (1922) and Fankhauser. Even though some of the activities of this larva were subnormal, so far as its internal morphology was concerned (Fankhauser, 1938*a*), the effects of the haploidy were not too serious.

It would be safe to conclude from the observations of Fankhauser on this single *Triton* larva that the morphological and physiological abnormalities which have been common to the majority of haploids need further investigation. The present experiments were undertaken, therefore, with the purpose of obtaining a number of advanced haploid larvae for a further study of haploid morphogenesis, and of examining the possibilities of experimental treatment of some of the serious physiological symptoms of haploidy in these animals.

The observations to be presented in this paper describe the animals which were obtained and the attempts to reduce the edematous swelling

of the body which appeared during the development of most of these larvae. Microscopical studies on the haploid animals will be published later.

The experiments were performed at Syracuse and many of the observations were carried out at the Marine Biological Laboratory, Woods Hole. A preliminary report of these experiments has already been published (Kaylor, 1939).

Material and Methods

Eggs of *Triturus pyrrhogaster* were selected for use, since, on the basis of Fankhauser's (1937) experiments on merogony with these eggs, it was believed that this species offered greater possibilities of extended haploid development than would others.

Eggs were obtained by anterior pituitary implantations. Development of the unoperated eggs was always normal. The female chromosomes were removed from the egg shortly after fertilization by means of a glass needle and small pipette (see puncturing method of Kaylor, 1937). All subsequent development then took place with only the male set of chromosomes. Control eggs, punctured to one side of the female nucleus, developed normally. These gave diploid embryos and larvae. Operated and control eggs were placed in a small dish of sterile modified Ringer solution,[1] and kept at a temperature of 20° to 22° C. until the animals had reached advanced larval stages (disappearance of yolk from the gut). From then on, the larvae were reared at room temperature varying between 22° and 26° C. Larvae were fed on *Enchytraeus*.

The corrosive sublimate, picric, acetic mixture of Michaelis was used for fixing yolk-laden embryos and larvae. Feeding larvae were fixed in Bouin's. In the sectioned material, Harris' hematoxylin was used for a nuclear stain and eosin or orange G in clove oil as a counter stain.

Observations

General Results (Table I)

Sixty-six out of 76, i.e., 87 per cent, of the punctured eggs of *Triturus pyrrhogaster* developed. The majority of these eggs were allowed to complete their developmental possibilities. Approximately 50 per cent of the developing eggs made at least an attempt at gastrulation, while 27 per cent developed beyond the gastrula stage. In a similar series of experiments with *Triturus viridescens* (Kaylor, 1937), only 10 per cent of all segmenting eggs developed beyond the gastrula stage.

[1] Solution according to Holtfreter except that the $NaHCO_3$ is omitted and the NaCl is reduced by one-half.

This comparison is of importance in experiments of this type where the main interest is in obtaining advanced stages of androgenetic development. It is also of interest to note from these observations that species differences are apparently an important factor in the extent and normality of haploid development, since in these two experiments the same methods were employed and the eggs were approximately the same size.

TABLE I

Androgenetic development of eggs of Triturus pyrrhogaster

Stage Reached	
No cleavage	10
Early cleavage stages	2
Late blastula	32
Gastrula	14
Neural plate	1
Neural tube	4
Neural tube, eye vesicles	1
Gill buds, limb buds, heart beat	7
Larva (30 days), three finger buds	1
Larva (47 days), hind limb buds	1
Larva (60 days), hind limbs four toes	1*
Larva (117 days), partly metamorphosed	1†
Larva (120 days), beginning metamorphosis	1‡
Total	76

* Killed by accident, not preserved.
† Died but preserved.
‡ Preserved while still developing.

Later Stages of Development

Evidence of Androgenetic Development.—The main interest of the present experiments was the group of 12 larvae which developed to stages ranging from the limb bud to a larva 120 days of age.

Seven of these larvae were preserved when they became edematous and failed to develop beyond the limb bud stage. Of the remaining five, which were the oldest larvae obtained, two were preserved because certain of their abnormalities were not compatible with further development, one (the oldest) was fixed while still developing, one was killed by accident some time after the first direct test of its chromosome condition, and one died at metamorphosis.

The evidence for the androgenetic development of these larvae is summarized in Table II. The retardation of development during gastrulation and neurulation has been established in other experiments (Kaylor,

1937; Porter, 1939) as a reliable criterion for the successful removal of the egg chromosomes.

TABLE II *

Evidence for androgenetic development of advanced larvae.

Stage of Fixation	Gastrulation and Neurulation	Pigment Cells	Chromosome Numbers
Limb bud, heart beat	Delayed	Small	—
Finger buds (30 days)	Delayed	Small	12±1
Hind limb buds (47 days)	Delayed	Small	12±1
Hind limbs (60 days)	Delayed	Large	33+2–3
Larva (117 days)	Delayed	Large	29+; 33+2–3
Larva (120 days)	Delayed	Small	12±1; 24±2

* The chromosome numbers are the highest and the lowest in 20 or more counts made from tail fin epidermis and from various organs of the body, except in the case of the two triploids. In the 60-day triploid, there were three counts in all and only from the tail tip; in the 117-day animal, eleven counts from various regions of the body.

Description of the Three Most Advanced Androgenetic Larvae.— 1. The first larva, preserved on the 47th day (Fig. 1*b*), exhibited the typical symptoms of permanent haploidy: stunted growth, blunt head and small eyes, small gills, small pigment cells, sluggish reactions. During earlier development it had been edematous, which will be referred to in the discussion of edema, but at the time of fixation it was entirely

Figures 1 and 2, photographs of two of the oldest androgenetic larvae obtained: a haploid (Fig. 1), a haploid-diploid (Fig. 2). Figs. 3 and 4, photographs of two stages in the development of the haploid-diploid larva. Figs. 5, 6, 7, photographs of three stages in the development of the third advanced androgenetic larva: the triploid which died on the 117th day.

FIG. 1, haploid larva 47 days of age (*b*), diploid control of same age (*a*). Tail tip of both had been clipped. 4 ×.

FIG. 2, haploid-diploid larva 120 days of age (*b*), diploid control of same age (*a*). Tail tip of both had been clipped. *ca* 2 ×.

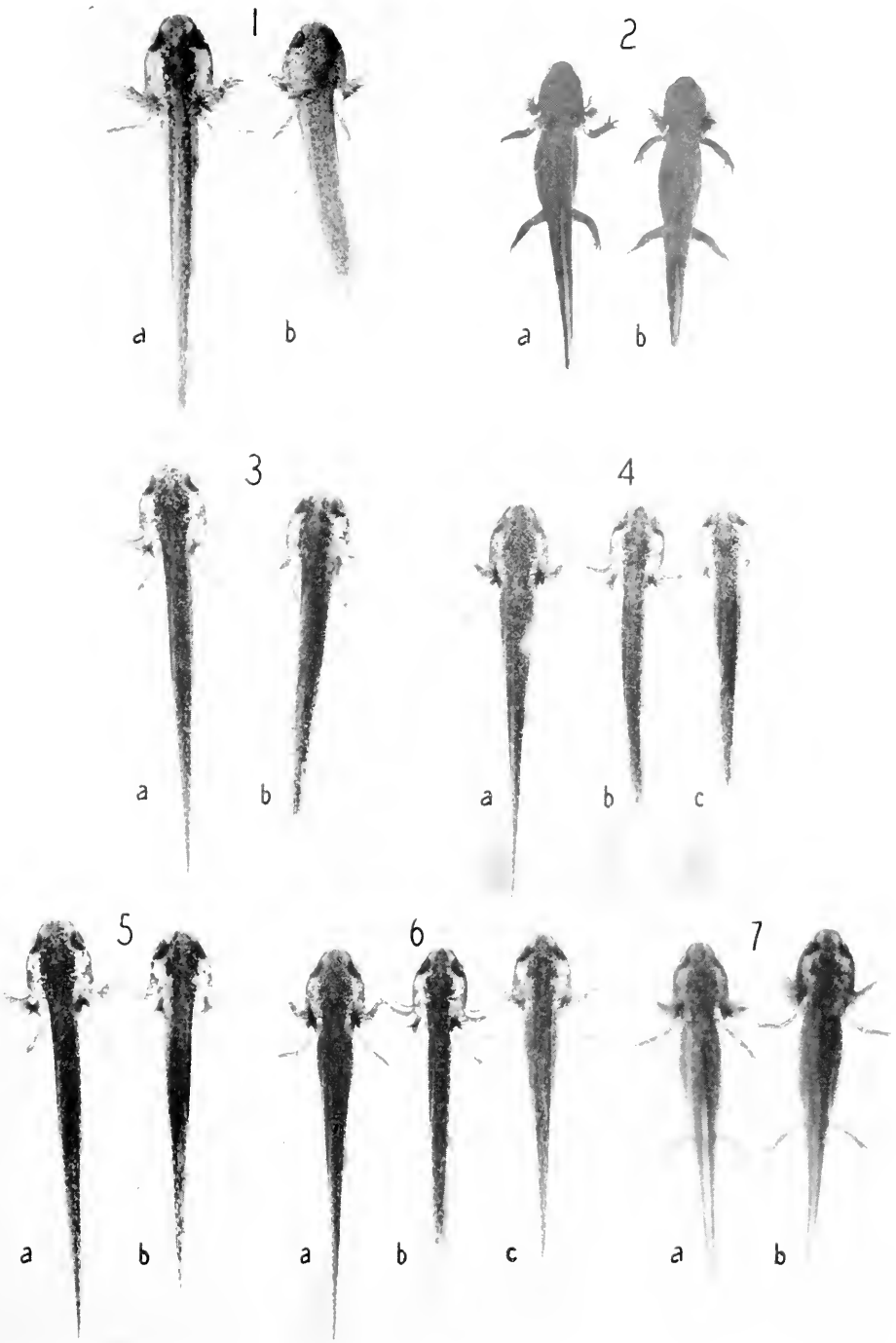
FIG. 3, the same haploid-diploid larva on the 21st day (*b*), diploid control of same age (*a*). Tail tip of *b* had been clipped. 4 ×.

FIG. 4, the same haploid-diploid larva on the 44th day (*c*), a diploid control about 3 days younger (*b*). Tail tip of both had been clipped. Diploid control (*a*) of same age as haploid-diploid larva. 2 ×.

FIG. 5, the triploid larva on the 21st day (*b*), a diploid control of same age (*a*). Tail tip of *b* had been clipped. 4 ×.

FIG. 6, the triploid larva on the 44th day (*c*), a diploid control about 3 days younger (*b*). Tail tip of both had been clipped. Diploid control (*a*) of same age as the triploid larva. 2 ×.

FIG. 7, the triploid larva on the 64th day (*b*), a diploid control of same age (*a*) with tail tip clipped. 2 ×.



FIGS. 1–7.

free of edematous swelling of the body which is so disastrous to most haploids. It was finally preserved, after it had exhausted the yolk supply in its gut, because its lower jaw was deformed and the animal was unable to take food. Introduction of macerated food through the mouth was unsuccessful.

All chromosome counts made so far in the tail fin epidermis and sections of the body have given the haploid number.

The existence of this larva demonstrates that advanced haploid larvae can be obtained with this method from whole eggs of this species. With larger numbers of animals it is not improbable that a greater number of advanced haploid larvae could be produced.

2. The second larva, fixed on the 120th day (Fig. 2*b*), unlike the majority of haploids, was not appreciably reduced in size in comparison to controls of the same age (Fig. 2*a*). Some of its morphological characteristics were, however, similar to those of other haploids, as, for example, the blunt nose and small head. In its reactions to stimuli the larva was as normal as any of the controls. This has not been a characteristic of all the other pure haploids obtained by a number of investigators. There had been an edematous condition of the belly and heart region during the limb bud stage, but this disappeared of its own accord and did not recur.

In spite of the fact that at the time of fixation this larva was unlike most haploids, there was the following evidence that it had developed with only the haploid male set of chromosomes: (1) A definite retardation in development at gastrulation and neurulation, which was evident in later stages (compare hind limb buds of this larva in Fig. 4*c* with a larva of the same age in Fig. 4*a* and with a control about 3 days younger in Fig. 4*b*). (2) Smaller pigment cells than those of controls (Figs. 3*b*, 4*c* compared to Figs. 3*a*, 4*a*, 4*b*). (3) The haploid chromosome number in ten nuclei of the tail tip epidermis of the young larva. Also the majority of epidermal nuclei (Fig. 8*a*) were smaller than diploid nuclei of controls (Fig. 8*b*).

However, when the entire animal was sectioned, preliminary chromosome counts in five different tissues showed that the larva was a mixture of haploid and diploid cells. Further investigation is needed to determine whether all organs are haploid-diploid. It seems probable, then, that regulation from haploidy to diploidy, which has been observed so frequently in parthenogenetically developed frog tadpoles (Parmenter, 1933, 1940; Porter, 1939; Kawamura, 1939), does occasionally occur in androgenesis in the newt.

Comparison of this larva (Fig. 2*b*) with the purely haploid larva (Fig. 1*b*) demonstrates, in part at least, the effect of haploidy-diploidy

upon the extent and normality of development. There is still the possibility, also, that a more normal morphological condition, which was independent of chromosome numbers, was obtained in this older larva.

3. A third larva died on the 117th day and was preserved. It had not completely metamorphosed. It was slightly larger than any of the controls of the same stage of development, possessed no apparent morphological irregularities, and was normally vigorous in all its reactions. No photograph was taken after the animal's death. However, in a photograph taken on the 64th day (Fig. 7*b*) it is seen that the larva appeared to be anatomically perfect and, even at this time, larger than controls of the same age (Fig. 7*a*). All larvae had been fed the same amount of food.

This larva was triploid, even though there was the suggestion that the animal had developed by means of androgenesis. The first prepara-

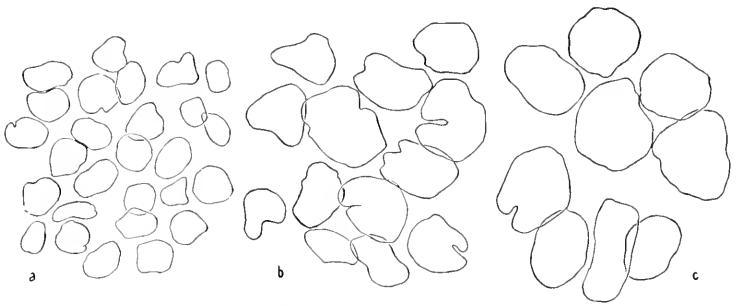


FIG. 8, camera lucida drawings of nuclei of epidermis cells of the haploid-diploid larva (*a*), of a diploid control (*b*), and of the triploid larva (*c*). 400 ×.

tion of tail fin epidermis offered 4 chromosome counts, all of which could be accepted as the triploid number. All nuclei in that piece of epidermis, a few of which are shown in Fig. 8*c*, were larger and less numerous than the diploid nuclei of controls (Fig. 8*b*). In a preliminary examination of sections of the entire larva, it was possible to make 7 chromosome counts from five different tissues (mesenchyme, cloacal epithelium, peritoneum, liver, heart). These counts were all approximately the triploid number, i.e., from $29+$ to $33+2-3$ (the triploid number being 36). It would appear, then, that this larva is entirely triploid and adds the eighth species to the list of Amphibia in which triploidy has been demonstrated.[2]

The evidence suggesting the origin of this triploid larva by means

[2] See review of literature by Fankhauser, 1938*b*; also see Kawamura, 1939, and Fankhauser, 1939.

of androgenesis is based on the following condition: a delay in development as compared to controls of the same age. The retardation of development, which was evident during gastrulation and neurulation, was still noticeable in the fore limb stage of the young triploid larva (Fig. 5*b* compared to Fig. 5*a*). Also the hind limb buds of this larva (Fig. 6*c*) were delayed in comparison to controls of the same age (Fig. 6*a*) and to controls about 3 days younger (Fig. 6*b*). Delayed development became less noticeable as the larva increased in age (Fig. 7*a* compared to Fig. 7*b*). The pigment cells were as large as or larger than those of the controls (as may be seen by magnifying Figs. 5*b*, 6*c* compared to Figs. 5*a*, 6*a*, 6*b*).

FIG. 9. Edematous haploid larvae (*a*, *b*, *c*, *d*, *e*, *f*, *g*) and a control (*h*) of the same age. Note the difference in size of the pigment cells of the haploid and diploid larvae. 4 ×.

There is no evidence as to the origin of triploidy in this case, but it must have occurred very early in development since only triploid chromosome numbers have so far been obtained in the entire larva.

Experiments to Reduce Edema.—The seven larvae which ceased development during the limb bud stage (Fig. 9, *a*, *b*, *c*, *d*, *e*, *f*, *g*) were edematous in the tissues of the heart and belly region. They were much smaller than controls (Fig. 9*h*) of the same age.

In two cases the edema began during the gill bud stage when pigment cells had appeared, the heart was beating, and Y-shaped blood islands were formed in the yolk region. In the other animals it appeared slightly later, at a time when circulation was established.

The development of the circulatory system of these larvae appeared to be abnormal, since in all cases there were localized accumulations of blood cells which persisted even after circulation had started in the rest of the body. This observation suggests that venous connection between the vitelline veins was not established, as Porter (1939) found in edematous frog larvae. The heart beat was normal. The pronephros

was probably established in all these larvae since the pronephric mound could be seen on the body surface. It is well established at this time in normal *Triturus pyrrhogaster* and venous connections of the cardinal veins are established in connection with the tubules. This suggests the functional activity of the tubules, since Armstrong (1932) has shown that in the fish, *Fundulus,* pronephric tubules become functional as soon as venous connections have developed in relation with the tubules.

As the edematous areas appeared they were opened under sterile conditions and the fluid allowed to escape. Then the animals were placed in sterile Clarke-Ringer solution.

In 6 cases, the edema returned within 12 to 24 hours in just as severe form as previously. The bloated areas were opened again and the larvae placed in sterile hypertonic salt solution but still the edema persisted. It gradually destroyed mesoderm and endoderm cells of the belly region and invaded the pericardial and head spaces. The heart action gradually became weaker until it was necessary to preserve the animals.

In the 7th larva (Fig. 9*a*), the edema returned in milder form and the animal lived for four days longer than the previous ones. However, its heart action gradually weakened and it was necessary to fix the animal. The head and fore limb region had differentiated slightly beyond the other six larvae.

The larva which was preserved on the 47th day (Fig. 1*a*) was also treated in the same manner for an edema which developed during the gill bud stage when circulation had been established. The condition disappeared completely and at the time of fixation it had not recurred.

A histological examination of all these larvae is in progress.

Discussion

The Production of Advanced Larvae

These results demonstrate that the majority of punctured eggs of *Triturus pyrrhogaster* made at least an attempt at development, and that a fairly high percentage of the segmenting eggs reached advanced stages of development.

It was also indicated that haploids of *Triturus pyrrhogaster* developed better than haploids of *Triturus viridescens.* Both species of eggs were practically the same size and were treated with identical methods. There are probably different factors in one species which determine the susceptibility of the developing eggs of that species to the effects of haploidy. Such variations between species (and genera) have been pointed out by other writers (P. Hertwig, 1923; Fankhauser, 1937; Porter, 1939).

However, the present experiments have produced no purely haploid

larvae which could equal the degree of differentiation obtained in the haploid *Triton* larva of Baltzer and Fankhauser. The reasons for this failure must await histological studies of the larvae which were obtained. There was the promising indication, on the other hand, that proper treatment of haploid larvae at critical periods in their development might possibly aid in lengthening the life of certain individuals.

One haploid-diploid larva was obtained which was apparently a normal individual since it developed to metamorphosis with no difficulty. It was pointed out that the success of extended development in this case may have been due to: (1) the normal number of chromosomes in many of its cells which may have affected physiological activities, and (2) a more normal morphological condition which occurred independent of the chromosome number.

Two triploid larvae were obtained in these experiments. All chromosome counts made so far in 5 different tissues of one larva have been triploid. The evidence for triploidy in the other larva is based solely on chromosome numbers and cell size in tail tip epidermis. The delay in development which was observed in both of these larvae might have been evidence of androgenesis. In this case, the triploid number of chromosomes must have originated in some manner from the sperm nucleus before first cleavage, because all chromosome numbers so far determined have been triploid. In order for this to have occurred it might be that the male haploid set of chromosomes divided once and then in some way one of these two sets divided again.

On the other hand, the delay in development might have been brought about by an entirely different set of circumstances: it may be that the female nucleus was left in the egg, retaining the second polar body (thereby giving a diploid female nucleus), and was then fertilized by the sperm nucleus. This would have produced a triploid embryo. It is impossible on the basis of the evidence at hand to say which of the two methods of origin of these triploid larvae was the most likely one.

Attempts to Reduce Edema

All haploids of these experiments suffered from edema at varying stages of development. In the majority of cases it was impossible to reduce this condition by the use of various concentrations of salt solutions. In only one larva was the edema permanently reduced by this treatment; in this case it is possible that the success was due primarily to a concomitant regulation of certain internal structures, such as the circulatory and kidney systems. Preliminary studies on the sections of this larva have indicated that this may indeed have been the case. Suc-

cessful treatment by these methods in any haploid larva, then, would depend upon the chance that certain internal structures are normally and even hyperplastically developed.

One other larva, the haploid-diploid, was edematous at an early period. The condition disappeared voluntarily. It is possible that further histological study of this larva may give a clue to the factors concerned in its improvement.

Aside from the observations on the living animals that the circulation was abnormal, no adequate explanations for the persistence of edema in these larvae can be advanced until the histological examination has been completed.

Summary

1. Eighty seven per cent of the punctured eggs of *Triturus pyrrhogaster* began development.

2. Advanced haploid larvae were obtained. Nine haploid larvae developed to stages ranging from the limb bud to a swimming larva 47 days of age. One haploid-diploid larva had begun to metamorphose at the time it was fixed.

3. Advanced androgenetic larvae can be obtained more readily in *Triturus pyrrhogaster* than in *Triturus viridescens: 27* per cent of the segmenting eggs developed beyond the gastrula stage in *Triturus pyrrhogaster* as compared to 10 per cent in *Triturus viridescens.*

4. Edema was the most common and serious abnormality associated with haploidy.

5. In the majority of cases it was impossible to reduce the edematous condition of the larvae with varying concentrations of salt solutions.

6. Two triploid larvae were obtained from punctured eggs. These larvae appeared to be entirely normal and slightly larger than the diploid larvae. This is the eighth species of amphibian in which triploidy has been observed.

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COMPARISON OF THE CHROMATOPHOROTROPIC ACTIVITY OF INSECT CORPORA CARDIACA WITH THAT OF CRUSTACEAN SINUS GLANDS[1]

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For a number of years it has been suspected from their histology that the corpora allata and the corpora cardiaca of insects have endocrine functions. Finally, in 1934, Wigglesworth demonstrated that tissues in the head of *Rhodnius* were responsible for humoral substances which were concerned with molt and metamorphosis, and later, in 1936, that growth and reproduction were also controlled to some extent by hormones. The corpora allata were suspected to be the source of the hormones concerned. Fraenkel (1935) demonstrated that a substance arising in the head of certain fly larvae was responsible for pupation in these forms. Pfeiffer (1939), by extirpation experiments, has shown that the gland concerned in the control of the activity of certain cells of the oviduct of the grasshopper was definitely the corpus allatum, as had been strongly suspected to be the gland concerned with the functions investigated both by Wigglesworth and by Fraenkel. Hanström (1936, 1937, and 1938) in a series of papers reported that the heads of insects, like the eyestalks of crustaceans, secreted a substance highly active in modifying the state of concentration and dispersion of crustacean chromatophores. In crustaceans Hanström had presented good support of the hypothesis that the sinus gland of the crustacean eyestalk was the source of the chromatophorotropic material. He was, however, unable to conclude just what was the source of the apparently comparable material from the insect head.

The greatest part of the work upon the insect endocrine glands has concerned the corpora allata. As yet no definite function has been ascribed to the corpora cardiaca. Recent work of Pfeiffer (1939) has given her some reason for suspecting that at least in *Melanoplus,* the corpora cardiaca or some structure located in the same region might be more directly concerned with molting in this form than are the corpora allata.

In the light of the work by Brown and Cunningham (1939) which

[1] This research was supported in part by a grant from the Graduate School of Northwestern University.

presented evidence that the sinus gland of *Cambarus* was directly concerned with control of molting in this form, and that of Brown (1940) showing that the sinus gland is also the eyestalk gland which produces chromatophorotropic hormones, it became interesting to discover whether in the insect as well, the same two functions (molt control and chromatophore activation) were to be found in a single gland. Were this true, then it would be the corpora allata to be first suspected since these were most generally believed to be the organs of the insect concerned with development.

Experimental

For these experiments the cockroach, *Periplaneta americana*, was used as the source of the insect tissues and the crayfish, *Cambarus immunis*, was the source of the crustacean tissues. After trying a number of methods of testing the action of various tissue extracts of the insect body upon crustacean chromatophores, it was finally decided to use isolated chromatophores as the test object. This practice was chosen inasmuch as it was possible to obtain great uniformity and sensitivity of response. The technic consisted of removing the carapace of a small crayfish (carapace length between 1.6 and 2.5 cm.) in the following manner: with fine scissors the carapace was cut from right to left just anterior to the cervical groove and then the scissors were inserted under the carapace in the median line at the posterior edge of the carapace in such a manner that the latter could be cut by a single stroke of the scissors into right and left halves. Each half was then removed by grasping the ventro-lateral edges of the carapace with forceps and lifting upwards, carefully tearing away the attaching muscles. Removing the carapace halves by grasping the dorsal cut edges invariably ruined the pieces. These two carapace halves, concave side uppermost, were placed in Syracuse watch-glasses, in sufficient Griffeth's solution to cover them. By rocking a sharp, round-pointed scalpel blade over them it was possible with a little practice to cut them up into a number of rectangular pieces (approximately 1×4 mm.) under a dissecting microscope. When viewed under a strong beam of light in an opaque white depression slide in a small quantity of Griffeth's solution with a compound microscope, it was possible to distinguish readily two types of chromatophores, red and white. These chromatophores were quite responsive to changes in the constitution of the bathing medium and retained this reactiveness for a number of hours. Furthermore, it was possible, by appropriate treatment of the crayfish which was to be the donor of the carapace, to obtain these two chromatophore types in almost any state of pigment dispersion or concentration. The sensitiveness of the isolated

chromatophores as test objects will become apparent in the experiments to be described.

Two difficulties arose in the use of these fragments of carapace in quantitative work. The first was that all of the chromatophores, either red or white, were not physiologically just alike. In the case of the darker red chromatophores there were those that were very sensitive to the dispersing and concentrating influences, and scattered in among these were smaller and paler red chromatophores which were somewhat less responsive. There appeared to be almost a complete series of grades of responsiveness. With the white chromatophores the situation was a little simpler since the few white chromatophores which were physiologically different from the majority lay in distinct patches. These patches were readily distinguishable since it was noted that these lay in the areas relatively free from red chromatophores and towards the dorsal portion of the carapace. By the proper selection of source of the test areas, these patches could be fairly well avoided. The second difficulty lay in the fact that at the greater dilutions of extracts tested, the chromatophores which were affected were more and more restricted to the peripheral region of the rectangular fragments, as if the material were diffusing inward from the cut edge.

In testing an extract sample, about 0.4 cc. of the sample was placed in a glazed porcelain depression and into this was placed one of the small carapace fragments. The general state of the red and white chromatophores was recorded at the beginning of the test and every fifteen minutes thereafter up to an hour. A control piece of carapace in Griffeth's solution was always kept for comparison. The state of the chromatophores was recorded in terms of a chromatophore index in which the chromatophore state at the time of any observation was expressed by a number ranging from one to five depending upon the degree of dispersion. A fully dispersed pigment mass was designated as 5 and a completely concentrated one as 1, with 2, 3, and 4 describing intermediate conditions. Due to the physiological differences of the chromatophores in a piece of integument, it was usually necessary to record a number describing the most fully dispersed chromatophores and another describing the most completely concentrated ones and then take the average value as the response to the extract tested.

The cockroaches used in the following experiments were all rather uniform in size ranging from 3.2 to 3.5 cm. in length (overall, excluding appendages). The crayfishes which served as donors of sinus glands varied in carapace length from 2.8 to 3.4 cm.

The Source of the Chromatophorotropic Hormones in the Insect Head

The first experiments were directed towards a localization of the source of the chromatophorotropic substance responsible for the results obtained by Hanström (1938). For this experiment the following tissues were carefully dissected out of the body of the cockroach: (1) brain; (2) frontal ganglion; (3) each of the three thoracic ganglia; (4) corpora cardiaca; (5) corpora allata. (Figure 1 shows a camera lucida outline of corpora allata and corpora cardiaca after dissection.) Each of the tissues was placed in a small porcelain depression. The brain was triturated and extracted in 0.8 cc. of Griffeth's solution; a single thoracic ganglion, the frontal ganglion, a single corpus allatum, and a single corpus cardiacum were each macerated and extracted in 0.4 cc. of Griffeth's solution. A piece of *Cambarus* carapace was added to

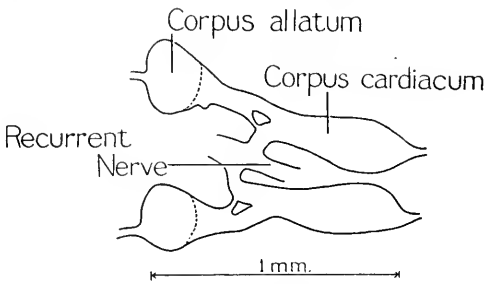

FIG. 1. A camera lucida outline of the isolated corpora allata and corpora cardiaca of the cockroach as seen from dorsal view.

each. Two Griffeth's solution controls were kept with each such experimental series. This procedure was repeated five times with the average results found in Fig. 2. The thoracic ganglia have been averaged together since there was not any perceptible difference found in the extracts of the three of them. It is noted that the only tissue of those tested which effected a concentration of the red pigment was the corpus cardiacum. On the other hand, it would appear from the data that brain and frontal ganglia were producing a concentration of the white pigment and that the thoracic ganglia were not doing likewise. Thus the concentrating agent of the brain and the frontal ganglion appears not to be a principle found in nervous tissue generally.

Brief experiments indicated that corpora cardiaca tissue extract was still strongly effective after boiling, and that the gland could be desiccated and stored for 24 hours without losing its capacity to concentrate red pigment when extracted.

The Size of the Cockroach Corpus Cardiacum

The cockroach corpus cardiacum was dissected out and outlined with the aid of a camera lucida. A rough approximation of its volume was made by constructing a clay replica of it and then reforming the clay into a sphere the volume of which was readily calculated from the diameter. Knowing the magnification of the model, it was easily found that

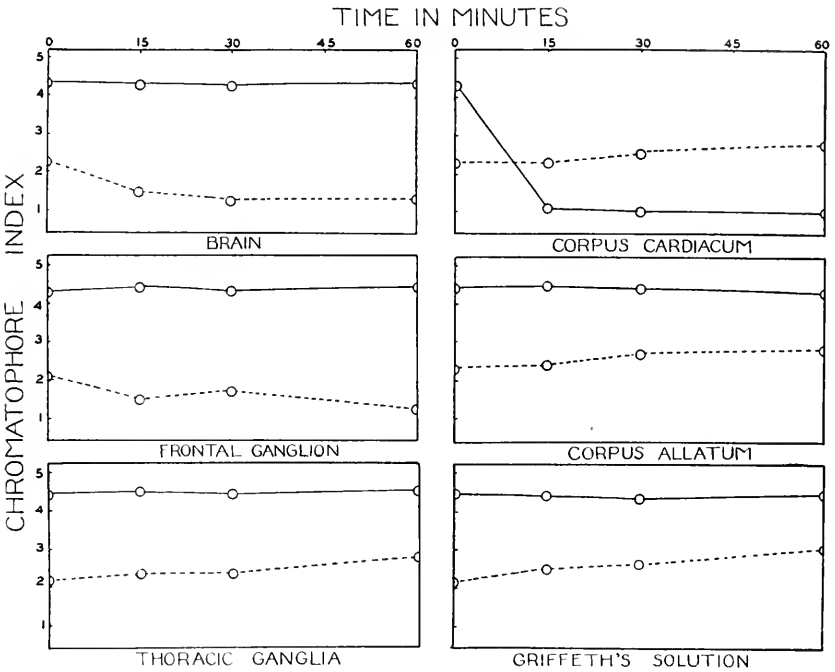

FIG. 2. The responses of red (solid line) and white (broken line) chromatophores of isolated pieces of crayfish integument to extracts of five organs of the cockroach body and to Griffeth's solution. A decreasing index with time indicates concentration of pigment and an increasing one, dispersion.

the volume of a single gland of the cockroach was about 0.019 mm.^{3}. The limits of the gland in making these measurements were determined by the extent of the bluish cast possessed by the gland when viewed in an intense beam of light. The glandular tissue appears more bluish than the neighboring nervous tissue and somewhat less transparent. In this respect, this gland resembles in appearance the sinus gland of the crayfish and other crustaceans.

On the basis of this rough determination it appeared that the corpus

cardiacum of our cockroaches was about half the volume of the crayfish sinus gland (Brown, 1940).

The Activities of Cockroach Corpora Cardiaca and Crayfish Sinus Glands as Seen in a Dilution Series

A long series of dilutions was made of each of the corpora cardiaca and the sinus glands and the effect of each member of the series was

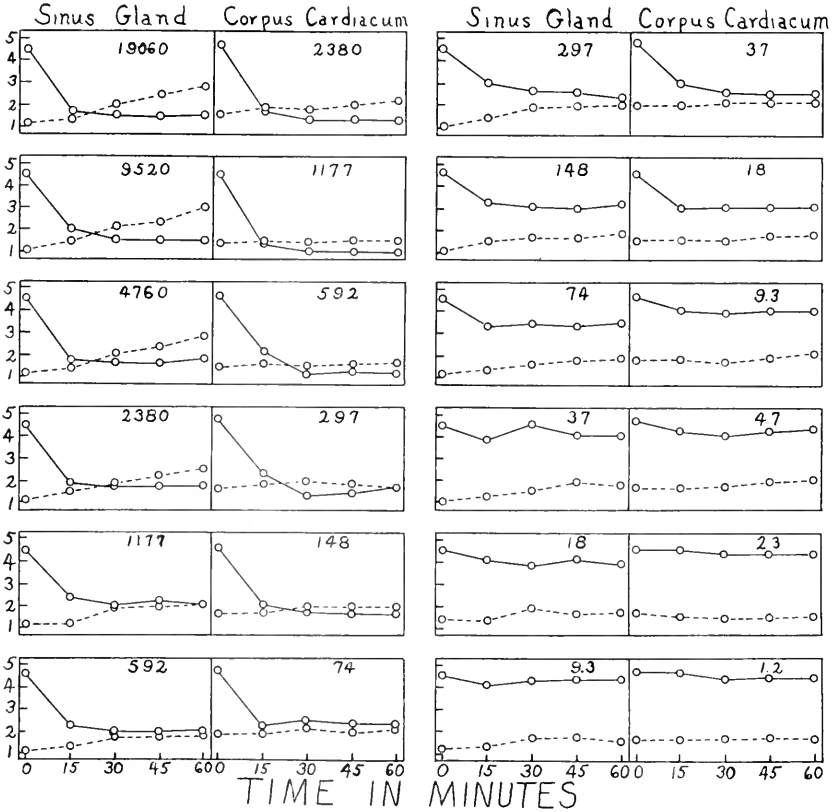

FIG. 3. The effect of dilution of the extracts of the insect corpora cardiaca and crustacean sinus glands upon red and white chromatophores. Red chromatophore responses are indicated by solid lines and white, by broken lines. The numerals along the ordinates are the average chromatophore indices. The numbers enclosed within the frames give the number of units of gland (by volume) to 2×10^8 units of solvent.

assayed in the usual manner upon isolated chromatophores. With such a method it was possible to discover the relative differences in the potency of the two tissues and at the same time to distinguish qualitative from quantitative differences in their activities.

For this experiment the corpora cardiaca were removed, permitted to dry briefly in a small mortar and then were triturated thoroughly in 0.8 cc. of Griffeth's solution. Half of this was used as the first sample to be tested and then the remainder was diluted to half the concentration and then half of this latter was set aside for testing. This procedure was repeated seventeen times, or in other words until the concentration was one part (by volume) of gland tissue to 2,760,000,000 parts of Griffeth's solution. It is important to point out that the dilutions were not made in the same vessel in which the glands were originally ground. Thus the results could not be influenced by a continued extraction of the original macerated tissue which might have adhered to the ground glass bottom of the mortar.

Exactly the same procedure was applied to the sinus glands of a crayfish. Two glands were originally extracted in 0.8 cc. of Griffeth's solution and diluted in steps with the concentration of each step being half that of the preceding one. Again here, seventeen such dilutions were made. Calculating on the basis that the volume of the sinus gland is about double that of the corpora cardiaca in animals of the sizes used, the maximum dilution obtained was one part gland to 1,380,000,000 parts of Griffeth's solution.

The above two experiments were repeated five times each. The greatest dilutions effective upon the red pigment for the experiments in which the corpora cardiaca were used were one part gland tissue by volume to:

11,050,000	parts	Griffeth's	solution
172,000,000	"	"	"
43,000,000	"	"	"
172,000,000	"	"	"
43,000,000	"	"	"

The average of these values is 88,200,000 parts of Griffeth's solution. In contrast to these, the last effective dilutions of the sinus glands fell at one part gland tissue to:

5,400,000	parts	Griffeth's	solution
2,700,000	"	"	"
43,000,000	"	"	"
1,347,000	"	"	"
21,600,000	"	"	"

The average of these values is 14,810,000 parts of Griffeth's solution. From this one would conclude that if the red pigment-concentrating substance is the same in the two glands the corpus cardiacum tissue con-

tains roughly six times as much active substance per unit volume as the sinus gland.

Figure 3 shows the comparative effects of dilutions of the corpora cardiaca and sinus glands upon the red and white chromatophores. A comparison of these two series yields some very interesting conclusions. (1) There is no significant effect of the extract of the corpora cardiaca upon the white chromatophores, while an extract of the sinus gland definitely dispersed the white pigment. (2) Volume for volume the corpora cardiaca tissue appears to be about eight times as effective upon the red chromatophores as the sinus gland.

Discussion

One interesting thing that these experiments indicate is that the crutacean sinus gland produces a concentration of the red pigment and simultaneously induces a dispersion of the white. At first this would appear to be a contradiction to the findings of Brown (1935) that eyestalk extract caused a concentration of the white when injected into eyestalkless *Palaemonetes*. The explanation of the difference is undoubtedly to be found in the great difference in the amount of eyestalk tissue involved in these two instances. Extracts of many crustacean central nervous organs is effective in concentrating white pigment.

Extracts of the central nervous system [2] of the crayfish are very effective in producing a concentration of both the red and the white pigment. In this we see a qualitative difference between the sinus gland and the nervous system since the former dispersed the white pigment. Although all parts of the crustacean nervous system are more or less effective upon both red and white pigment, the findings in this research indicate that the cockroach does not possess a red concentrating principle in any part of its brain or thoracic nerve cord or in its frontal ganglion, but does possess a white concentrating substance in its brain and frontal ganglion.[3] Thus it would appear that the chromatophorotropic substances which we have found in the nervous system are not materials of general distribution in the nervous tissue of all forms and hence are more likely to have a specific function of a humoral nature within the bodies of those forms in which they occur. The white chromatophores of most crustaceans respond to background changes and all the evidence at hand indicates that they are controlled in this reaction by hormones. Now, as far as we know, the only source of a concentrating principle for

[2] This effect is apparently not due to acetyl choline since experiments using a wide range of concentrations of this latter substance failed to give similar results.

[3] We have failed to find a red concentrating principle in the annelid nervous system.

this pigment is in the central nervous system, while an antagonistic (dispersing) substance is formed in the sinus gland. A comparable dual control of the black pigment in the telson and uropods of *Crago* is found (Brown and Ederstrom, 1940) in which the sinus gland produces a concentrating substance and part of the central nervous system (commissural ganglia) produces a dispersing substance. We are therefore led to suspect that here in the arthropods the nervous and endocrine mechanisms are more closely associated morphologically than in the higher vertebrates. Some of the hormones are produced by definite glandular bodies apart from the nervous system, while others are produced by cells intimately associated with nerve cells or perhaps possessing the dual function of secretion of hormones and conduction of nerve impulses.

The dilution experiments give us no reason for supposing that the red chromatophore-affecting materials from the insect corpora cardiaca and that from the crustacean sinus gland are not chemically the same. The material from the corpora cardiaca loses its effectiveness upon dilution at the same rate as that from the sinus gland. Furthermore, both withstand boiling and both retain their activity for some time in desiccated tissues. If these substances are the same, then a separate substance which disperses white must be present in the sinus gland.

Summary

1. The corpora cardiaca are the organs in the head of the insect which are responsible for the formation of a substance which produces a strong concentration of the pigment of the red chromatophores of crustaceans.

2. The corpora cardiaca still have an effect upon isolated red chromatophores of *Cambarus* when the gland is extracted in 80,000,000 times its volume of Griffeth's solution.

3. Neither the corpora cardiaca nor the corpora allata have any perceptible effect upon the white chromatophores of *Cambarus;* the latter gland is also without effect upon the red chromatophores.

4. The crustacean sinus glands still have an effect upon isolated red chromatophores when extracted in about 14,000,000 times their volume of Griffeth's solution.

5. The sinus glands of crayfishes produce a dispersion of the pigment of the white chromatophores of *Cambarus*.

6. The crustacean sinus gland and the insect corpora cardiaca differ qualitatively from one another with respect to their action upon the white chromatophores. With respect to the red pigment-affecting principle, the two glands are similar in that the rate of loss of effectiveness

with dilution is the same for both, boiling the extracts inactivates neither, and both glands retain their effectiveness for some time in a desiccated state.

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THE INDEPENDENT ORIGIN OF AMPHIBIAN RED CELLS AS SHOWN BY DIFFERENTIAL SUSCEPTIBILITY TO X-RAYS

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Knower (1907) described amphibian embryos in which normal blood cells and posterior circulatory channels developed after the heart anlage had been surgically removed. Interestingly enough, he noted in a single sentence the superficial similarity of the surviving operated embryos to certain X-rayed specimens. Federici (1926), Goss (1928), and Slonimski (1931) have reported successful surgical removal of the single large ventral blood island of early amphibian tadpoles and the subsequent development of normal, pulsating hearts and blood vessels without any circulating blood cells. The paper of Goss (*loc. cit.*) includes a translation of the account of the normal development of the blood island as given by Brachet (1921) and an excellent bibliography on the subject. Goss concluded that, since the excision of the blood island resulted as described above, "The angioblast concept cannot be applied to amphibians." In his experiments, performed with fine iridectomy scissors, it was necessary to remove a large part of the ventral region of the embryo with attendant loss of yolk, anomalies of the digestive system, and a very heavy operative mortality.

Stockard (1915) was able to obtain teleost embryos in which the normal erythrocytes developed in the blood islands and normal pulsating hearts were formed, although immersion in weak alcohol solutions prevented the connection of the hearts with the veins and forced the embryos to develop without circulation of the blood.

These experiments showed the separable, mutually exclusive character of the anlagen of the erythrocytes and heart at the very early stages used but did not define their places in the scale of differentiation. Among tadpoles of *Amblystoma jeffersonianum*, developed from embryos X-rayed at the early neurula (Harrison normal series, No. 14) stage, I have found embryos in which the specific dissimilarity of the

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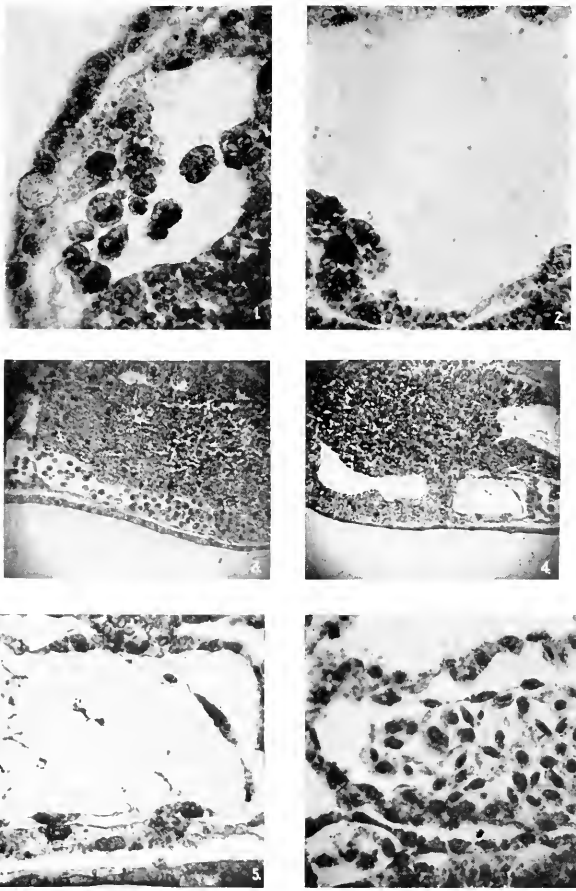
embryonic differentiation of these anlagen is indicated. In such embryos the potential blood island cells are relatively so sensitive to X-ray injury that they may be completely inhibited by relatively low X-ray exposures (1000 *r*), while the anlagen of the heart and endothelium are uninjured and subsequently develop in a normal manner except that the colorless fluid which circulates is devoid of blood cells.



Photographs by Dr. Luther Smith, Research Associate, Field Crops Genetics, University of Missouri.

FIG. 1. Part of ventral blood sinus from normal 8-day embryo. × 75.

FIG. 2. Part of heart, same embryo as Fig. 1. × 75.

FIG. 3. Part of sagittal section of 12-day normal embryo. × 18.

FIG. 4. Part of sagittal section of 12-day embryo which had received 1000 *r* on the 4th day. × 18.

FIG. 5. Heart from Fig. 4, enlarged. × 75.

FIG. 6. Heart of normal 12-day embryo. × 75.

One thousand *r* were insufficient to inhibit the formation of heart and endothelium at the same stage, or any of the 5 other stages exposed and studied, so that the differences appear to be both quantitative and qualitative. Figure 1 is a photograph of a part of a sagittal section of a normal, 8-day embryo near the median plane. Part of the ventral blood sinus, formed at the site of the ventral blood island, is shown and the characteristic spherical, yolk-filled primitive blood cells are seen floating in their fluid or plasma. Figure 2 is an enlargement of the heart of the same animal with definite, continuous endothelial lining but no blood cells. Figure 3 is from a sagittal section of an embryo 4 days older showing the heart cavity and ventral blood sinus confluent and the primitive blood cells in all regions of the system. Note that at the caudal (right) end of the sinus part of the ventral blood island remains a compact mass. It can be readily observed that the ventral blood sinus is here supplied with an endothelium continuous with that of the heart (left). In Fig. 4 is shown a section of a 12-day embryo which had received 1,000 *r* 8 days earlier. The large heart (right) has a continuous endothelium but there is no endothelium lining the posterior cavity (left) in the blood island region and there are no blood cells. Figure 5 is an enlargement of the heart shown in Fig. 4 and Fig. 6 is a normal heart of the same age. It is crowded with red blood cells, many of which have changed from the primitive spherical type to the more advanced shape typical of the mature erythrocyte.

It appears from the study of these embryos and others from the same series that the anlage of the ventral blood island differs sufficiently from that of the heart and endothelium to allow X-ray destruction of the former without apparent harm to the latter. The bloodless condition was found in 9 of the 45 embryos studied. In 21 others the red cells were reduced in number and retarded in development.

In a separate series which received only 500 units of X-ray some specimens killed on the 20th day still show bloodless endothelial-lined hearts and unlined ventral blood sinuses not connected with the hearts.

It is concluded that the heart and endothelium of *Amblystoma jeffersonianum* arise from cells specifically distinct from the cells which produce the primitive blood cells. The difference is not a difference of degree of development or of position in the body but a true physiochemical difference. The red cells lost through inactivation of the blood island by X-rays are not replaced from any other source during the prehatching period.

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QUANTITATIVE STUDIES UPON SOME BLOOD CONSTITUENTS OF HELIX POMATIA

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The living activities of *Helix pomatia* are in many ways dependent upon the weather conditions. During the cold months, from about October or November to March or April, the snails hibernate, closing their shells by secretion of the calcareous epiphragma, and receiving their energy from stored reserve substances. During the warm months the intensity of their activity depends largely upon the amount of rain falling in any particular year, the snails feeding and being active only in moist surroundings with the period of reproduction lasting usually from June to August. In a previous investigation (v. Brand, 1931) it was shown that the chemical composition of the body of *Helix* shows characteristic differences at various times during the annual cycle. It seemed of interest to study some of the blood constituents, in order to test whether similar regular changes occur here.

Material and Methods

The animals, *Helix pomatia,* were purchased from a dealer in Franconia. They were kept in an enclosure in a garden and fed regularly with lettuce and cabbage leaves. Broken pieces of *Helix* shells were provided to ensure a sufficient calcium supply. The snails lived under these conditions indefinitely with few animals dying. Every year, however, a new lot of freshly collected snails was added.

Almost every month of the years 1933 to 1936 the blood of two lots of snails (10 to 12 animals each) was collected by puncture of the lateral vein. The following determinations were performed: dry substance by drying a sample at 100° C., total inorganic substances by incineration of dry substance, P according to Embden (1921), Ca according to Holtz (1934). The Ca analyses were usually performed on an incinerated blood sample, although preliminary tests showed that a direct precipitation of the calcium with oxalate, as practised in analyses on human blood, is also possible. In the latter case the snail blood has to be diluted with 4 to 5 volumes of distilled water, because of the large amounts of calcium present. Cl was determined according to Austin and van Slyke

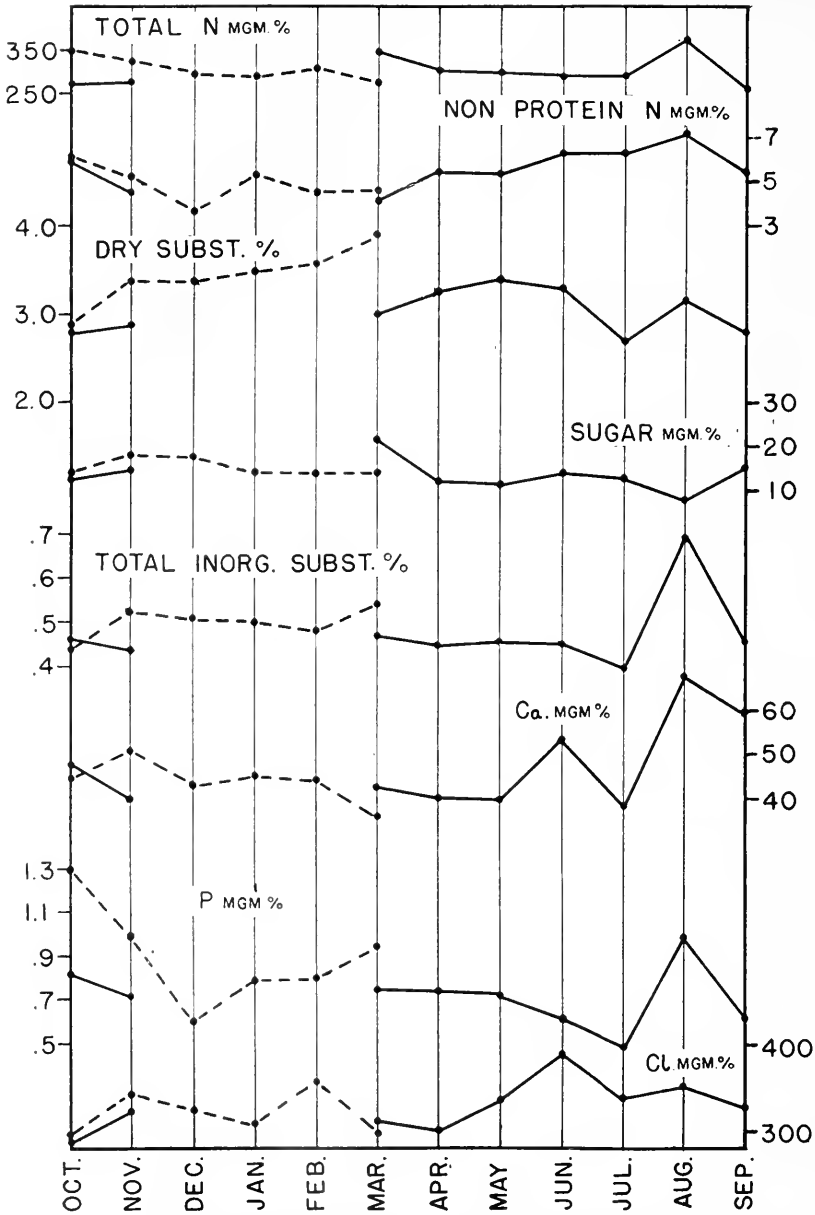

FIG. 1. Monthly averages of blood constituents of *Helix pomatia*. Solid line = Active life. Broken line = Hibernation.

(1920, 1921), sugar according to Hagedorn and Jensen (1923), total N and non-protein N according to a micro-Kjeldahl procedure. The values reproduced below for non-protein N are considerably lower than the figures of Wolf (1933), who reported values of roughly 20 mgm. per cent. This difference must be attributed to an incomplete precipitation of the proteins by Wolf. Dr. Wolf has since repeated his non-protein analyses in the laboratory of one of the authors (F. H.) and found the same low values that we give below. We used for the precipitation of the proteins a phosphomolybdenic reagent as prescribed by Bang (1927). Slightly higher values were found in preliminary experiments by using a mixture of tri-chlor-acetic acid with sodium-wolframate or, according to Folin, sodium-wolframate with ⅔ normal sulfuric acid.

TABLE I

Average values and maximal and minimal values of blood constituents of Helix pomatia.

	Average		Maximum		Minimum	
	Active life	Hibernation	Active life	Hibernation	Active life	Hibernation
Dry substance, *per cent*	3.1	3.4	3.9	4.0	2.0	2.4
Inorganic substances, *per cent*	.48	.50	.76	.63	.24	.40
Cl, *mgm. per cent*	331	322	450	395	220	265
P, *mgm. per cent*	.73	.91	1.80	1.50	.25	.36
Ca, *mgm. per cent*	48	44	133	82	28	7.9
Sugar, *mgm. per cent*	14	16	35	30	4	7
Total N, *mgm. per cent*	302	310	530	290	180	200
Non-protein N, *mgm. per cent*	5.6	4.9	14.6	8.4	4.4	3.0

Results

Fairly large variations of the monthly averages were apparent for all substances and they were in general decidedly more pronounced during the time of active life than during the period of hibernation (Fig. 1). Contrary to the findings reported previously for several constituents of the whole snail bodies, no significantly different trend of the blood constituent curves could be found during the different periods of the year. The extent of the variations present is demonstrated in Table I, where the maxima and minima both for the period of hibernation and of active life are shown. It is apparent that the minima are lower and the maxima are higher during active life. This is not surprising, since it is to be expected that with varying food and water intake during this time, the

composition of the blood will be more variable than during the period of hibernation.

It should be noted that many of the substances tested (i.e. total inorganic, Ca, P, total N and non-protein N) showed a peak for their monthly averages in August (Fig. 1). With the exception of non-protein N the highest absolute values (Table I) occurred in this month. The explanation lies in the fact that August is the hottest and dryest month of the year. A certain concentration of the blood may be expected to result from these climatic conditions. In agreement with this

TABLE II

Mean values of blood constituents of Helix pomatia during the formation of the epiphragma. Extreme values are given in parentheses.

	Active life	During process of secretion of epiphragma	After formation of epiphragma
Dry substance, *per cent*	2.35 (2.04–2.68)	3.08 (2.60–3.92)	3.41 (2.78–3.73)
Inorganic substances, *per cent*	.42 (.34–.56)	.50 (.42–.58)	.45 (.40–.48)
Cl, *mgm. per cent*	290 (184–391)	302 (270–354)	311 (263–348)
P, *mgm. per cent*	.73 (.56–1.0)	.94 (.67–1.1)	1.24 (1.1–1.4)
Ca, *mgm. per cent*	38.4 (35.5–43.0)	38.5 (32.5–49.5)	37.9 (34.5–44.0)
Sugar, *mgm. per cent*	15 (7–21)	17 (9–22)	16 (11–21)
Total N, *mgm. per cent*	272 (187–306)	327 (290–358)	358 (318–382)
Non-protein N, *mgm. per cent*	5.5 (4.2–8.1)	5.8 (4.6–7.3)	5.9 (3.3–8.4)

assumption is the fact that the blood sugar was found to be very low in August—in a dry month the chances are small that a snail will feed much. Curiously enough, Cl and total dry substance were neither especially high nor low during August.

It should be kept in mind that many irregularities of the curves will be due to accidental causes, for example whether the animals had or had not received rain during a few days, or even hours before the analyses. Such conditions can change the picture from year to year. It might be mentioned as an example that in one year the monthly maximum for total N was found in August; in another year the animals analyzed in the same month gave values similar to the lowest of this whole series.

In order to test whether or not a significant difference between the blood composition during the periods of hibernation and of active life exist, the monthly averages of both periods have been averaged. In this way the accidental causes due to weather conditions are thought to be eliminated for the most part. It is quite apparent (Table I) that the values for both periods are identical. It seems likely that these values, which are based on a large number of determinations, represent the normal average level of these substances in the blood of *Helix*. It seems necessary to assume that in *Helix*, as in higher animals, a regulation of blood constituents is present. But the levels are not as fixed as in the latter, as demonstrated by the variations mentioned above.

It has been stated previously (v. Brand, 1931) that about half the inorganic substance occurring in a snail in autumn is used for the formation of the epiphragma. At the same time much water is lost from the body. It seemed of interest to study the blood composition during this period and in the spring when the epiphragma is shed. The animals intended for these determinations were kept under close observation in the fall of 1935 and 1936. Three groups of individuals were analyzed: (1) animals leading an active life, (2) animals in the process of secreting the epiphragma, and (3) animals having just completed its formation. The three lots were analyzed on the same days. They had therefore been subjected to the same climatic conditions before the analyses. In the spring of 1936 and 1937 the blood composition of snails at the end of the hibernating period and after having shed the epiphragma was investigated in a similar way.

It is apparent that during formation of the epiphragma (Table II) the dry substance content of the blood rises. This is due largely to an increase of the protein concentration, as evidenced by the considerable increase in total N. Non-protein N and sugar were practically unchanged.

Both the total inorganic substance and two of its components, Cl and Ca, showed almost no change. P showed a more distinct increase. The fact that the inorganic substance showed no greater changes during the formation of the essentially inorganic epiphragma, is rather remarkable. The epiphragma is secreted by glands located at the edge of the mantle. An accumulation of inorganic substances there is known to occur long before the epiphragma is actually secreted. However, Barfurth (1883) showed that the inorganic substances of the liver are greatly reduced after the formation of the epiphragma. Obviously, it is to be expected that they were transported to the mantle edge by the blood. That no rise in the blood occurs, may be explained on the assumption

TABLE III

Mean values of blood constituents of Helix pomatia at the end of the hibernation period. Extreme values are given in parentheses.

	Before shedding the epiphragma	After shedding the epiphragma
Dry substance, *per cent*	3.87 (3.32–4.20)	2.85 (2.56–3.05)
Total inorganic substances, *per cent*	.53 (.48–.60)	.42 (.34–.51)
Cl, *mgm. per cent*	302 (270–319)	274 (248–286)
P, *mgm. per cent*	1.37 (.95–2.00)	1.07 (.45–2.00)
Ca, *mgm. per cent*	31 (23–37)	30 (18–40)
Sugar, *mgm. per cent*	13 (7–16)	35 (29–47)
Total N, *mgm. per cent*	386 (319–588)	327 (300–372)
Non-protein N, *mgm. per cent*	6.3 (4.8–9.2)	4.7 (3.4–6.5)

that these substances are deposited in the glands at the same rate as that at which they are mobilized in other tissues.

As soon as the epiphragma was removed in the spring and the snail resumed an active life, the concentration of most substances tested

TABLE IV

Effect of moisture and food on blood constituents of Helix pomatia.

	Lot 1 (moist surroundings + food)	Lot 2 (moist surroundings, no food)	Lot 3 (dry surroundings, no food)
Dry substance, *per cent*	2.98	3.02	3.06
Inorganic substances, *per cent*	.60	.63	.67
Total N, *mgm. per cent*	295	270	258
Non-protein N, *mgm. per cent*	1.9	1.5	1.3
Dry substance of snail body minus shell, *per cent*	22.6	21.5	22.8

dropped more or less (Table III). This is doubtless due to the fact that a snail sheds its epihragma in general only if the moisture in the surroundings is high, i.e. after a rain. It then has an opportunity to take in water at once. A corresponding observation concerning the total

body tissue has been published previously (v. Brand, 1931). It is curious to note, and as yet not explainable that the Ca content of the blood showed no change comparable to that of the other substances. The sugar content of the blood of free snails was found to have risen considerably. This may be interpreted as an alimentary hyperglycemia, since animals having shed the epiphragma begin immediately to feed.

Some experiments were performed in order to ascertain whether any blood constituents can be influenced readily under experimental conditions. They are of a rather preliminary nature, but are recorded here since we are at the present time not able to follow them up.

A short period of dryness, such as frequently occurs in nature, seems not to change the constitution of the blood to a marked degree. Fifty snails were divided into three lots as follows. Lot 1 was kept on moist

TABLE V

Blood sugar of Helix pomatia fed with cabbage soaked in 2 per cent dextrose solution.

Time	Blood Sugar *mgm. per cent*	Condition of Animals
0	8	Before feeding
1 hour	25	During "
2 hours	30	" "
3½ hours	49	" "
5½ hours	22	2 hrs. after feeding
7 hours	19	3½ " " "
9 hours	17	5½ " " "

filter paper with access to cabbage, lot 2 on moist filter paper but starving. Lot 3 was kept without food in an atmosphere dried by calcium chloride. The snails of lots 1 and 2 were active, those of lot 3 remained inside their shell most of the time, some closing the opening with a thin membrane. The blood composition after 5 days under these conditions is summarized in Table IV. There is no difference between the three lots.

The sugar content of the blood, on the other hand, can be influenced easily. A lot of snails was kept for some days without food or water. Then they were offered cabbage soaked in 2 per cent dextrose solution. They were allowed to feed for 3½ hours, following which they were again starved. At certain intervals during and after the feeding period the blood of some of the snails was collected and analyzed for sugar. A pronounced alimentary hyperglycemia was observed during the feeding period (Table V). The blood sugar sank slowly again as the snails

TABLE VI

Calcium content in normal and regenerating pieces of shell and in blood of Helix pomatia.

Lot	Days after removal of original piece	Ca Content		
		Removed piece *per cent total weight*	Regenerated piece *per cent total weight*	Blood *mgm. per cent*
1	0	—	—	38
2	7	44	10	94
3	14	34	33	42
4	24	36	32	34
5	34	35	?	32

were again starved, but it was still higher at the end of the experiment than at the beginning.

The last experiment deals with the question of whether the blood calcium rises when the snail is forced to regenerate a part of its shell. A fairly large piece of the shell was removed from some snails, and its Ca content was analyzed. After certain periods the Ca content of the piece regenerated and that of the blood was determined (Table VI). The Ca content in the regenerated piece reached the normal level after about two weeks. The value in lot 2 measured 7 days after the operation seems to indicate that the calcium content of the blood rises considerably in the early regeneration period. This is a contrast to the findings reported above concerning the formation of the equally calcareous epiphragma. Such a difference is not surprising. The formation of the epiphragma is a physiological process, whereas the regeneration of a large piece of shell is hardly a process to which the snail body is especially adapted. Small repairs, of course, occur frequently. Snails, for example, which had sealed together broken pieces of their shell, were fairly frequent in our material. We doubt, however, that under natural conditions snails would survive large defects to their shell.

Summary

1. Monthly analyses on different blood constituents of *Helix pomatia* showed that their concentration may vary considerably.

2. The mean values, gained from a large number of analyses, indicate, however, that there is no significant difference between the blood composition during the time of hibernation and that of active life. This is due to the fact that most, if not all, of the variability is due to climatic variations which balance out if observations are made over a long period

3. In snails forming the epiphragma the blood proteins and P were found in a certain concentration, but not the other inorganic substances.

4. The blood constituents become diluted by intake of water in snails shedding the epiphragma.

5. Short periods of dryness do not materially change the blood composition.

6. An alimentary hyperglycemia can be produced easily by feeding sugar to the snails.

7. In the early stages of shell regeneration the blood calcium is higher than normal.

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THE DISTRIBUTION OF INTERMEDIN: FIRST APPEARANCE OF THE HORMONE IN THE EARLY ONTOGENY OF RANA PIPIENS[1]

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Introduction

Extensive experimental work on the cytological picture of the pituitary gland, and statistical studies of the shifts in cell population of the adult organ have contributed materially to our knowledge of the physiology of the hypophysis. In the lower vertebrates, detailed studies of the histogenesis of the pars buccalis, which would be of significance morphologically and physiologically in relation to the sequence of events in ontogeny, have been relatively few; in the mammals, such investigations have been more extensive, although frequently made in a more fragmentary manner. Admittedly, integration of our knowledge of histogenesis with development of function in the pituitary gland is desirable, not only as an end in itself, but for explaining the phylogenetic significance of specific hormone effects. The chromatophorotropic hormone, intermedin, secreted by the pars intermedia, is interesting when considered in this respect. This hormone plays an important rôle in the regulation of physiological color changes in the lower vertebrates; in mammals, on the other hand, although large amounts of intermedin are present in the hypophysis, no definite function can be ascribed to this hormone, despite several attempts to investigate its part in the mammalian system.

In an earlier report (Kleinholz and Rahn, 1940) a method of assay for intermedin was developed, using the hypophysectomized lizard, *Anolis carolinensis,* as a biological test object. An attempt in that study to correlate the production of the chromatophorotropic principle in the pars anterior of the hypophysis of the chicken with a specific cell type did not yield thoroughly conclusive results because of the complexity of the material. The opinion was ventured that a physiological study of the ontogeny of intermedin might be more revealing, especially if studied in association with the appearance of cell types in the embryonic glands.

[1] These studies on the distribution of intermedin were aided by a research grant from the Permanent Science Fund of the American Academy of Arts and Sciences.

This report is an account of the ontogeny of the chromatophorotropic hormone of the pituitary gland in the frog, *Rana pipiens.*

Materials and Methods

Eggs of the frog, *Rana pipiens,* were obtained throughout the period of investigation by injecting mature females with extracts of triturated pituitary glands. The eggs thus secured were fertilized and allowed to develop at a room temperature of approximately 20° C. At various periods in the development, samples consisting of 250 to 300 individuals were removed for assay.

As is commonly known, even when precautions are taken to insure uniform environmental conditions, the eggs of a mass show considerable variation in the times at which they reach given embryological stages (Pollister and Moore, 1937). Table I shows the stages of the larvae at

Table I

Stages in the early ontogeny of R. pipiens taken for assay.

Stage	Age after fertilization	Stage number of Pollister and Moore
Four-cell	4 hours	4
Neural fold	52 hours	14
Tail-bud	96 hours	17
7-mm. larva	144 hours	20

the time they were taken for assay, and their age from the time of fertilization. In addition, unfertilized eggs from the ovary of a normal (uninjected) female killed in November were taken for testing.

The material to be used for assay was rinsed rapidly in two changes of pure acetone to remove excess water, after which the eggs and embryos were given a final change of 50 cc. of pure acetone for 24 hours. The unhatched stages (ovarian eggs, 4-cell and neural-fold batches) were dissected from their jelly or connective tissue capsules under acetone. The heads of the larvae in the tail-bud and 7-mm. stages, after the preliminary drying in acetone, were ablated (Fig. 1) and kept separately from the decapitated bodies which were to be used in the preparation of control extracts. The acetone-dried material was then dried in air at 40° C. for 3 days. At the end of this period the various embryonic stages were ground to a powder in a mortar and stored in a desiccator until used in the preparation of extracts.

The extracts were prepared by treating weighed samples of the dried material with N/10 NaOH, heating to the boiling point, neutralizing

with N/10 HCl against phenolphthalein as an indicator, and diluting to desired concentrations (from 3.6 to 9.0 mg. dry weight of powder per 1.0 cc.) with cold-blooded Ringer's solution. The stock extracts thus prepared were placed in ampules and capped, then immersed in boiling water for 15 minutes, after which they were stored at 1° C. No preservatives were added.

The prepared extracts were injected as 0.2 cc. samples intraperitoneally into each of 10 hypophysectomized lizards, and the degree of dispersion of the pigment within the dermal melanophores was measured on a numerical scale of five stages of the total color range (see Kleinholz and Rahn, 1940). For the bionomics and physiology of metachrosis in this animal, reference may be made to an earlier study (Kleinholz, 1938).

Observations

The data obtained from these experiments are arranged in Tables II and III. In Table II are shown the concentrations of the prepared extracts and the amounts of injected material from the embryonic and larval stages. Table III shows the melanophore responses of the groups of test animals to injection of the various extracts.

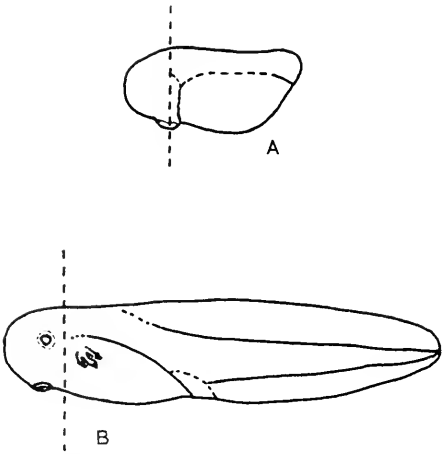

Fig. 1. (*A*) The tail-bud larva and (*B*) the 7 mm. free-swimming larva. The dashed vertical lines show the amount of cephalic material used for the preparation of the extracts.

It is evident from an examination of these tables that ovarian eggs, eggs in the four-cell stage and in the neural-fold stage contain no intermedin and therefore have no effect on the dermal melanophores of the test animals, even though injected as extracts of comparatively high con-

centration. The first positive response of the melanophores was obtained with extracts prepared from heads of larvae in the tail-bud stage. The average response for 30 injected animals was 1.3 ± 0.9 on the scale of the chromatic range. As defined in the previous study (Kleinholz and Rahn, 1940), the *Anolis* unit (A.U.) for intermedin is that weight of pituitary powder or its equivalent which will evoke an average stage 1 response in a group of 10 injected test animals. Since each of the injected animals received the equivalent of 4 heads of larvae in the tail-bud stage (Table II), each head therefore contains approximately 0.32 A.U. of intermedin. Control injection of extracts prepared from the de-

TABLE II

Concentration of the prepared extracts and the amount of tissue injected into each test animal.

Extract no.	Nature of extracted material	Number used	Dry weight	Conc. of extract (wt. dry powder in 1.0 cc.)	Amount of tissue injected into each animal
			mg.	*mg.*	
1	Ovarian eggs	250	252.9	7.2	1.4 eggs
2	Four-cell stage	210	207.2	7.2	1.4 eggs
3	Neural-fold stage	260	265.6	9.0	1.8 eggs
4*a*	Heads of tail-bud larvae	240	43.6	3.6	4.0 heads
4*b*	Bodies of tail-bud larvae	240	184.6	3.6	0.9 body
5*a*	Heads of 7-mm. free-swimming larvae	264	40.5	3.6	4.8 heads
5*b*	Bodies of 7-mm. free-swimming larvae	264	198.6	3.6	0.9 body
6	Heads of hypophysectomized 7-mm. free-swimming larvae	50	—	—	5.0 heads

capitated bodies of these larvae, in equal amounts and concentrations, gave no detectable response. Similarly, extracts prepared from the heads of 7-mm. free-swimming larvae upon injection yielded an average response of 1.7 ± 1.1 for 30 lizards, indicating an approximate intermedin content of 0.35 A.U. per head in this developmental stage. Considering the fact that these are biological tests made with comparatively crude materials, the values for the amount of intermedin in the heads of the two larval stages are in very good agreement and indicate the sensitivity of the test in the assay of minute quantities of biological material. Control injections made with extracts prepared from the decapitated bodies of these 7-mm. larvae were completely without effect.

These results where the first detectable amounts of intermedin in the

frog larva appear during the tail-bud stage of development are readily correlated with the appearance of the hypophyseal primordium as an ingrowth of the buccal ectoderm. To eliminate the remaining possibility that some cephalic structure outside of the hypophyseal primordium was responsible for the intermedin effect, frog larvae in the tail-bud stage were hypophysectomized according to the method of Smith (1916). These animals were allowed to grow to 7-mm. in length, at which time the heads of 50 larvae were separated from the bodies and prepared as

TABLE III

Melanophore responses of the test animals to injection of extracts.

Extract number	Dry weight of powder injected as extract into each animal	Number of animals injected	Response
	mg.		
1	1.44	9	0
2	1.44	10	0
3	1.80	10	0
3	1.80	10	0
4*a*	0.72	10	0.9±0.7
4*a*	0.72	10	1.2±1.0
4*a*	0.72	10	1.9±0.9
4*a* (average)	0.72	30	1.3±0.9
4*b*	0.72	10	0
5*a*	0.72	10	1.7±1.1
5*a*	0.72	10	1.6±1.2
5*a*	0.72	10	1.7±1.2
5*a* (average)	0.72	30	1.7±1.1
5*b*	0.72	10	0
6	0.72*	10	0

* Five heads were injected into each animal. From Table II, this amount is estimated to weigh approximately the figure given.

before. The extracts prepared from these hypophysectomized heads contained no detectable amounts of intermedin.

DISCUSSION

An attempted correlation between ontogeny of intermedin and the cytological differentiation of the pituitary gland in *R. pipiens* is seen to be pointless. The first detectable traces of intermedin in the embryology of the frog occur at the tail-bud stage, where the ectodermal hypophysis has only recently become invaginated to lie below the infundibulum. Although at this stage the cells of the pituitary demonstrate a well-initiated physiological differentiation, there is no cytological differentiation. His-

tological examination of the hypophyseal primordium from the tail-bud larva reveals embryonic ectodermal cells containing scanty, non-staining cytoplasm, frequently with pigment (melanin) granules which have presumably been derived from the egg pigment by passive enclosure during cleavage. No chromophillic granules are present and the pars intermedia is not differentiated. Kerr (1939) describes a very similar condition for the newly-hatched larva of *R. temporaria*. According to this author, the first eosinophiles appear in the anterior pituitary of larvae which are 11–13 mm. in length, while "in the intermediate (lobe) of the frog a few scattered basophiles are to be found" in the 32 mm. tadpole which has hind and fore legs and tail fully developed.

Several studies of the pituitary gland have been made integrating cytological development with the development of physiological activity. Outside of this report and the work of Kerr (1939) there are, however, only a few other studies on the differentiation of the pars intermedia and the appearance of detectable amounts of intermedin. Snyder (1928) detected qualitatively the presence of intermedin in pituitary glands from pig embryos of 30 mm. crown-rump length. Both Maurer and Lewis (1922) and Nelson (1933) found that the first secretory granules in the pars intermedia of the pig appeared in embryos 175 mm. in length. The former authors, however, connected this histological differentiation with the appearance of the pressor principle of the pituitary gland.

Intermedin is thus seen, from the results cited above, to be one of the earliest hormones formed by the pituitary gland. Certainly, in the frog, the first detectable amounts of this hormone appear with the formation of the pituitary primordium. The physiological significance of this early appearance of intermedin is probably to be correlated with the onset in larvae of *R. pipiens* of physiological color changes about 10 days after hatching. The absence of metachrosis in the early larval stages (4–7 mm. length) of this species is undoubtedly due to the fact that morphological differentiation has lagged behind physiological differentiation; that is, the integumentary melanophores, the eye, and the optic pathways to the brain and pars intermedia have not yet become fully established.

Summary

1. Quantitative assays for the first appearance of intermedin in larvae of *R. pipiens* were made, using the hypophysectomized lizard, *Anolis carolinensis*, as test animal.

2. Intermedin is detectable with the establishment of the hypophyseal primordium as an invagination from the buccal ectoderm. The gland from larvae 4–7 mm. in length contains approximately 0.3 *Anolis* unit of intermedin.

3. Intermedin appears before cytological differentiation of the pituitary gland occurs.

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STUDIES ON THE TREMATODE FAMILY MICROPHALLIDAE TRAVASSOS, 1921

IV. The Life Cycle and Ecology of Gynaecotyla nassicola (Cable and Hunninen, 1938) Yamaguti, 1939

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Introduction

In the first of this series of studies the writer (1939) described *Cornucopula sippiwissettensis* gen. et sp. nov. from shore birds near Woods Hole, Mass. Since then, an investigation into the life cycle has shown that *Cercaria nassicola* Cable and Hunninen, 1938, is the larval stage of this trematode. Yamaguti (1939) established a new genus, *Gynaecotyla,* to include species formerly placed in the genus *Levinseniella* but having characters incompatible with those described for *Levinseniella.* Since Yamaguti's paper appeared while the writer's was in press, his generic name has priority; likewise, Cable and Hunninen's specific name has priority; therefore, the correct name of this microphallid trematode is *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Yamaguti, 1939.

Until the brief report by the writer (1939*a*) of the life cycle of *Cornucopula nassicola* (now *G. nassicola*), no complete life history of a microphallid trematode was known. Numerous reports have indicated larval stages in various genera of this group. Reference to the first three papers of this series (Rankin, 1939, 1939*b*, 1940) and to the papers of Hadley and Castle (1940) and Cable and Hunninen (1940) will eliminate unnecessary discussion here of the literature on this subject.

The present paper is an elaboration of the original brief report (1939*a*), presenting an experimental proof of the life cycle of *Gynaecotyla nassicola* with notes on the ecology of this parasite.

It is unfortunate that this genus must again be emended. But since several important characters are either interpreted wrongly or are mentioned as absent when actually they are present, a restatement of the generic diagnosis seems pertinent. In the first place, Yamaguti (1934, 1939) described a female genital sucker lying between the acetabulum and the cirrus or testis; this structure has been shown by the writer (1939) to be a second ventral sucker, with no connection whatsoever

with the genital system. Secondly, Yamaguti indicated that there is no receptaculum seminis or Laurer's canal; actually, these structures are present and can be found even in the metacercaria. Finally, several writers have included this genus in the family Heterophyidae. As will be discussed later, a study of the morphology and life history indicates that *Gynaecotyla* belongs to the family Microphallidae.

Gynaecotyla Yamaguti, 1939, char. emend.
Syn.: *Cornucopula* Rankin, 1939

General Diagnosis.—Microphallidae Travassos, 1921. Very small, pear-shaped trematodes with spiny cuticula; anterior end bluntly tapering, posterior end broadly rounded. Two ventral suckers located at beginning of posterior body third. Prepharynx, pharynx, and esophagus present; intestinal crura usually extend to posterior level of ventral suckers. Male copulatory organ dextral, consisting of a complicated muscular structure lying in the genital atrium; it is composed of two recurved horn-like projections, the tips of which usually bear spines; pars prostatica weakly developed. Uterus enters genital atrium at lateral edge of dextral acetabulum, close to the common genital opening. Large transverse cirrus pouch, containing bulbous seminal vesicle and coiled ductus ejaculatorius, lies anterior to the ventral suckers. Ovary sinistral, lateral to and on same level as ventral suckers. Testes symmetrical, just posterior to the ventral suckers. Uterine coils filling space behind testes; may extend in front of testes to anterior edge of suckers. Laurer's canal and receptaculum seminis present. Vitellaria follicular, in two compact groups, one behind each testis. Excretory bladder V-shaped; flame-cell pattern, 2 $[(2+2)+(2+2)]$. Adults in intestine and caeca of shore birds; metacercariae in crustaceans. Type species: *Gynaecotyla squatarolae* (Yamaguti, 1934) Yamaguti, 1939.

Syn.: *Levinseniella squatarolae* Yamaguti, 1934
Cornucopula squatarolae (Yamaguti, 1934) Rankin, 1939

Other species include:

1. *Gynaecotyla jägerskioldi* (Travassos, 1921)
   Syn.: *Levinseniella jägerskioldi* Travassos, 1921
   Cornucopula jägerskioldi (Travassos, 1921) Rankin, 1939
2. *Gynaecotyla simillimus* (Travassos, 1921)
   Syn.: *Levinseniella simillimus* Travassos, 1921
   Cornucopula simillimus (Travassos, 1921) Rankin, 1939
3. *Gynaecotyla aduncа* (Linton, 1905)
   Syn.: *Distomum aduncum* Linton, 1905

Levinseniella adunca (Linton, 1905) Linton, 1928
Cornucopula adunca (Linton, 1905) Rankin, 1939

4. *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Yamaguti, 1939
Syn.: *Cornucopula sippiwissettensis* Rankin, 1939
Cornucopula nassicola (Cable and Hunninen, 1938) Rankin, 1939

Figures and descriptions of all five species may be found in the writer's 1939 paper.

Materials and Methods

Living material was used as much as possible, because many structures, particularly gland ducts and flame cells, cannot be observed in preserved specimens. Neutral red was used successfully as an intravitam stain. Material for mounting whole was fixed in Conant's fixative (50 per cent Alc., 100 cc.; Formalin, 6.5 cc.; glacial acetic acid 2.5 cc.), stained in Grenacher's Borax-Carmine precipitated with HCl, and mounted in damar. Material for sectioning was fixed in Bouin's fixative, stained in Delafield's haematoxylin, and counterstained with eosin.

Snails from the infected locality were isolated in fingerbowls to obtain cercariae; crustaceans were examined for metacercariae. In order to obtain parasite-free hosts for experimental infection, snails and crustaceans from widely separated localities were isolated and examined. Young gulls, used as experimental definitive hosts, were taken from the nest when they were four or five days old and kept on a diet of fish and squid.

All drawings were made with the aid of the camera lucida, small details filled in by free hand. All measurements given below are in millimeters.

Field Observations

While studying the trematode parasites of shore birds in the Woods Hole region, it was observed that these birds were feeding largely, if not entirely, on crustaceans. Stomach contents invariably consisted of digested or partially digested specimens of *Talorchestia, Orchestia, Gammarus,* etc. The following crustaceans were collected and examined for metacercariae: *Talorchestia longicornis* (Say), *Orchestia platensis* Kröyer, *Chirodotea cacca* (Say), *Gammarus locusta* (Linn.), *Haustorius arenarius* (Slabber), *Crago septemspinosus* (Say), *Palaemonetes vulgaris* (Say), and *Virbius zostericola* S. L. Smith. In *Talorchestia longicornis* alone were trematode cysts found and these cysts contained metacercariae that appeared morphologically like the adult *Gynaecotyla*

nassicola found in the birds. These metacercariae were fed to young gulls and in a few days examination of these experimental hosts produced adult *G. nassicola,* thereby establishing the identity of the metacercariae.

The only snails occurring in abundance in this area are *Littorina littorea* (Linn.) and *Nassa obsoleta* (Say). Thousands of these were isolated. A tiny microphallid cercaria was found in *Nassa,* none in *Littorina.* This was identified as *Cercaria nassicola* described by Cable and Hunninen (1938, 1940). Experimental evidence has yielded positive proof of the identity of this cercaria as the larval stage of *Gynaecotyla nassicola.*

Experiments Proving the Life Cycle

Penetration of Cercaria into Crustacean Host

Several specimens of *Nassa obsoleta* shedding *Cercaria nassicola* were placed in fingerbowls with *Talorchestia longicornis,* since this was the only species of crustacean found naturally infected. Controls were kept at all times. Examinations were made at regular intervals of both experimental and control animals. Negative results were obtained. Most of the crustaceans were either killed by the snails or drowned. Since this cercaria is the only one of its kind in the locality, the negative results were puzzling. A study of the habits of *Talorchestia* indicated that this amphipod lives in the sand *near* the water line, not *in* the water. In fact, when a fingerbowl had sand in it rising above the water level, the talorchestiae always climbed out of the water on to the sand. This suggested another set of experiments. Three dishes, six inches in diameter and three inches deep, were used. Sand to the depth of one-half inch was placed in each dish with a small mound of it to one side. Water was added to cover the bottom sand, but the mound was left above the water level. Three specimens of *Nassa obsoleta* shedding cercariae were placed in each dish, along with ten *Talorchestia* from an uninfected locality. The water was changed morning and night by running sea water into the sand, thoroughly washing it. This simulated the diurnal changes occurring on the beach. One hundred per cent infection was obtained; the controls proved negative. The first crustacean was examined five days after exposure and contained nine tiny cysts. A corresponding increase in number and size of cysts was found with longer exposure. A maximum of 93 cysts, with an average of 22 per host, was obtained. In nature, an average of 6 cysts, with a maximum of 26, was found in 100 specimens of *Talorchestia* examined. The cysts are found free in the pericardial cavity, not embedded in tissues as is characteristic of other

members of this family (Cable and Hunninen, 1940; Hadley and Castle, 1940).

The method of penetration is quite different from that reported for *Spelotrema nicolli*. In the latter, the crab becomes infected in the water by sweeping the cercariae into the gill chamber where the larvae penetrate into the efferent veins. Since *Talorchestia* is not equipped with this type of respiratory mechanism, a different mode of entrance must be sought. Also, since these amphipods died when left in water alone, another method must be used. Cercariae without tails were found actively moving about in the branchial lamellae (Fig. 1) attached to the second gnathopods and first, second, third, and fourth periopods. Some were found boring through the tissues towards the dorsal pericardial cavity. These tiny cercariae, then, like so many of the microscopic sand-dwelling species, can live in the moist sand between the tides and can penetrate the amphipods as the latter lie quiescent below the surface. The sand- and mud-burrowing habit of the snail host also aids in completing this stage of the worm's life cycle.

Specimens of *Haustorius arenarius*, *Chirodotea cacca*, *Emerita talpoida*, and *Orchestia platensis* were exposed in a manner similar to that above. Not one of these animals became infected.

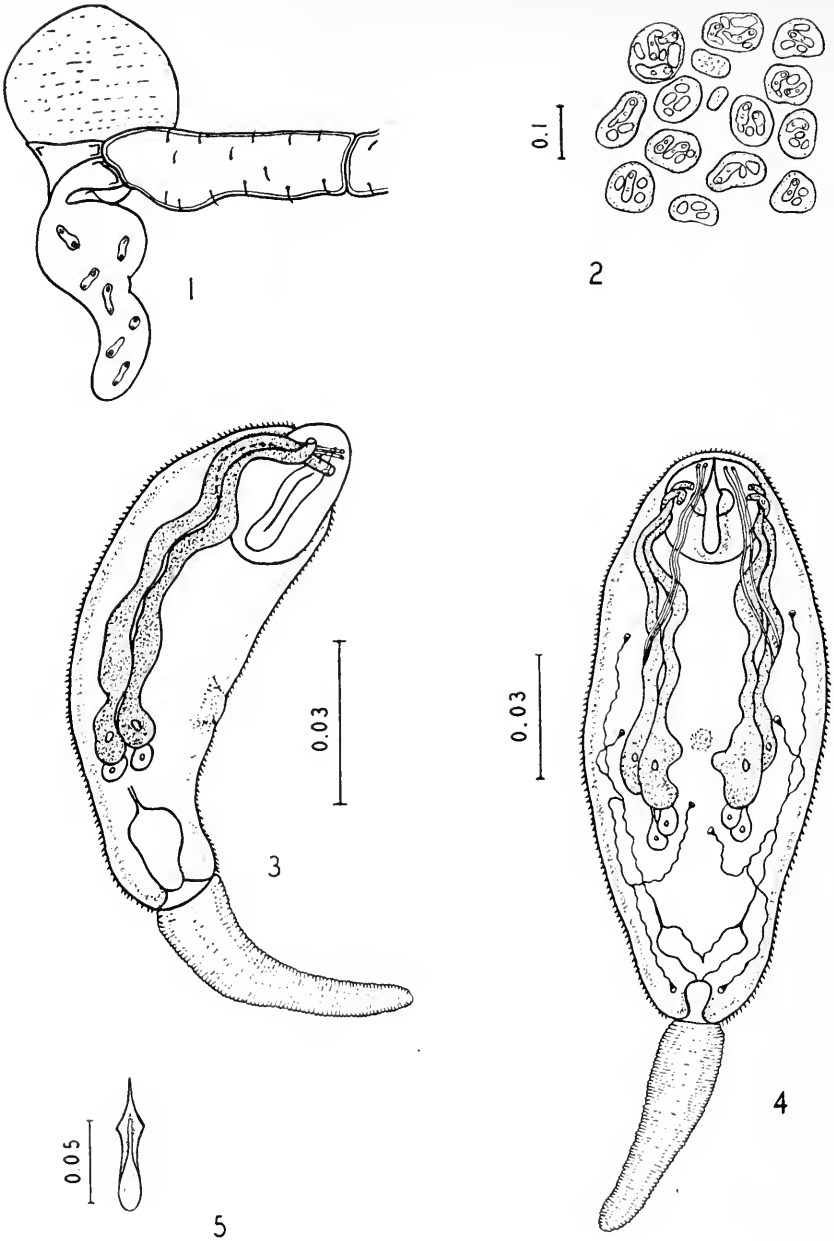
Infection of Definitive Host

Fifty mature cysts collected from naturally infected talorchestiae were fed to one young herring gull (*Larus argentatus*). Two days later the bird was killed and examined. Twenty-one immature adults of *Gynaecotyla nassicola* were obtained, some with eggs present in the uterus. Sixty-five cysts from experimentally infected talorchestiae were fed to a second gull, and 36 to a third. Thirty-five adults were obtained on examination of the former host seven days later, and 15 from the latter eight days later. Two control birds were negative for this species of trematode.

Description of Stages in the Life Cycle

Sporocyst (*Fig. 2*)

The cercariae develop in small oval, thin-walled sporocysts averaging 0.1 in diameter. Many specimens of *Nassa obsoleta* were found to be heavily infected, the livers completely riddled with these larvae. Very few cercariae or germ balls are present, a maximum of 8 cercariae, with an average of 6 being found. A study of sectioned material indicates that two or more sporocyst generations may occur.



EXPLANATION OF PLATE I

FIG. 1. Proximal end of second gnathopod of experimentally infected *Talorchestia longicornis*. Note tail-less cercaria that have penetrated the branchial lamella.

FIG. 2. Cross-section through sporocysts in the liver of *Nassa*.

FIG. 3. Lateral view of cercaria showing openings of glands, unusual thickness of gland ducts, position of stylet, and primordium of ventral suckers.

FIG. 4. Ventral view of cercaria showing arrangement of glands and excretory pattern.

FIG. 5. Stylet of cercaria.

Cercaria (*Figs. 3, 4, 5*)

Since Cable and Hunninen have described this cercaria well, only additions will be given here. *Cercaria nassicola* is a tiny xiphidiocercaria of the ubiquita group, averaging 0.2 in length. It is a slow but continuous swimmer when above the bottom; as soon as it touches the bottom, however, it moves actively over it. Touching the cercaria while it was swimming did not cause cessation of motion. No ventral sucker as such has been observed. In sectioned specimens, however, a small vacuole or cavity may be found in the region of the future suckers. Likewise, in some flattened specimens, a thickening in this region may be found. The number of glands and arrangement of ducts were found to be as described. Four flame cells were found on each side, in the formula $2[(1+1)+(1+1)]$. The stylet is quite large in comparison with the size of the oral sucker, measuring 0.023 in length. It is lancet-shaped anteriorly, bluntly rounded posteriorly, while an elongate keel protrudes a short distance ventrally.

Metacercaria (*Figs. 6, 7*)

The metacercariae occur free in the pericardial cavity of the crustacean. These roll out when the host is torn open, with no host tissue attached. In the smaller cysts, the stylet remains in position. As the metacercaria grows, the stylet is absorbed, not shed, for it was never found either in position or in the cyst cavity in the larger metacercariae. Remains of stylet glands were present in all larvae found. Most of the adult characters were found even in the youngest metacercariae examined: the two acetabula, the genitalia, and the convoluted walls of the intestinal crura. The cysts averaged 0.26 in diameter, never exceeding 0.29. The cyst wall averaged 0.015 in thickness. The body is covered with spines as far posteriorly as the acetabula. Large refractile glands are located beneath the cuticula of the whole body and render difficult observation of body systems, particularly the excretory system. The excretory bladder appears to be without a lumen, filled with large cells. The flame cell pattern is the same as that for the adult, $2[(2+2)+(2+2)]$.

The younger metacercariae are removed easily from the cyst but die very quickly. Older specimens, however, are removed with difficulty due to increased thickness of the cyst wall. These may be mounted in mammalian Ringer's solution and remain alive for several hours.

Adult (Figs. 8, 9)

Since the adult of this species has been adequately described in a previous paper (Rankin, 1939) only a few controversial details will be discussed. Yamaguti (1934, 1940) considered the second ventral sucker to be a female genital sucker located at the external opening of the uterus. In the present study, however, metacercariae of various ages, ranging from stages with only poorly developed to those with fully developed suckers, have been examined both alive and sectioned. In no case was any connection ever found between the uterus and this second sucker. In development and histological appearance it has every character of a true sucker. Figure 9 indicates the appearance of the two ventral suckers and their striking similarity is readily apparent. Perhaps phylogenetically this second sucker may have been derived from a genital sucker, having lost this former function and connection secondarily. However, the present evidence does not permit such a conclusion, but does indicate the acetabular nature of this structure.

A receptaculum seminis and Laurer's canal have been reported by Yamaguti and others as absent from genera considered here as in the family Microphallidae. The writer (1939, etc.), Cable and Hunninen (1940), and Hadley and Castle (1940) have shown that these structures do exist in species of *Levinseniella, Spelotrema, Maritrema,* and *Gynaecotyla.* A careful study of live material will probably show these structures to occur in other genera likewise, for only on living specimens have they been observed. They are so thin-walled that they collapse immediately on fixation. In *G. nassicola,* the receptaculum seminis and Laurer's canal may be discerned particularly well in immature specimens in which the uterine coils are not yet filled with eggs (Fig. 8).

The Egg and Miracidium (Fig. 10)

The structure and development of the miracidium are very difficult to follow due to its small size. The eggs measure 0.02×0.01 and are very numerous. In young worms the eggs are practically undeveloped;

Explanation of Plate II

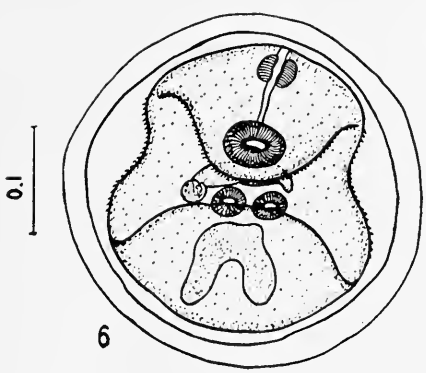
Fig. 6. Metacercaria within cyst. Note that even through the cyst wall the two acetabula may be observed.

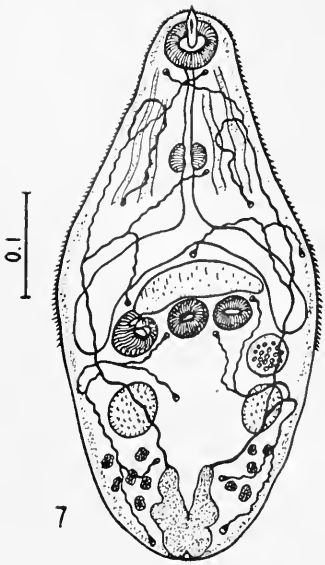
Fig. 7. Metacercaria removed from cyst wall. Stylet still present along with remains of the cephalic or stylet glands; genitalia fairly well developed; flame cell pattern shown.

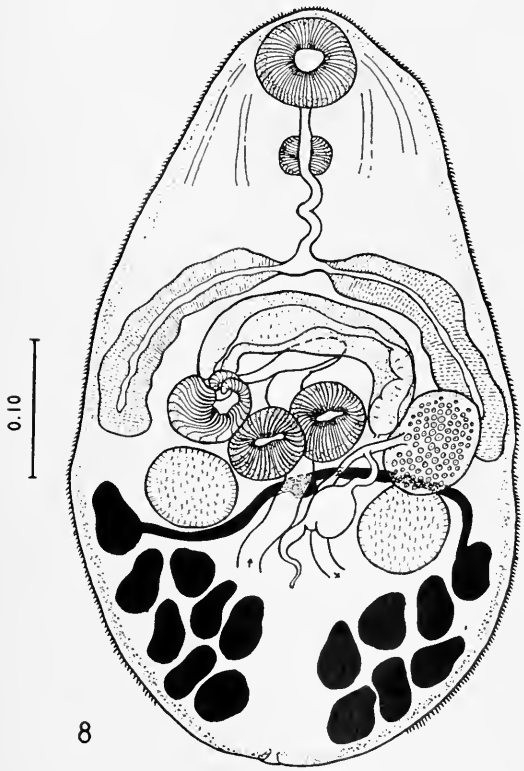
Fig. 8. Immature adult recovered from an experimentally infected gull. Remains of gland ducts still present. Note especially the two large, similar acetabula, and the large bulbous receptaculum seminis with a short Laurer's canal.

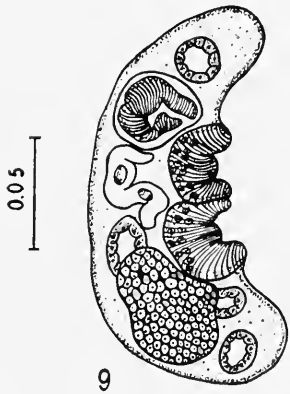
Fig. 9. Cross-section through the ventral suckers and the copulatory complex. The identities of the two suckers are easily discerned.

Fig. 10. Immature egg.









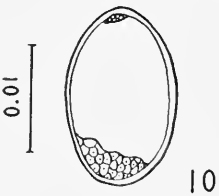


PLATE II

in older specimens, crowding prevents clear observation. Development in sea water or in mammalian Ringer's solution is very slow and no larva has been carried through to hatching. Consequently, the mode of penetration into the snail host has not been observed.

Ecology of Gynaecotyla nassicola

The relationship between the habits of host and parasite is well illustrated in the life cycle of *Gynaccotyla nassicola.* In a comparatively small area all three hosts occur in relative abundance. *Nassa obsoleta* literally covers the beach at the infected locality near the low tide zone. As the water recedes at low tide, the snails are exposed and crawl about on the sand or burrow beneath the surface. The crustaceans, likewise, emerge by the thousand from the sand at low tide and have been seen running across the damp surface in hordes. Plovers, sandpipers, and other shore birds may be observed running up and down the beach feeding on these amphipods. Other species of crustaceans usually remain buried in the sand or under drift weed along the water's edge. *Talorchestia* is the only one exposed so plentifully to the birds.

A study of the seasonal distribution of infection of *Nassa* with the cercariae of *G. nassicola* yielded the following results (the first figure after the date is the number of snails examined; the second figure is the percentage of infection): July, 1939: 717, 2.9; August: 481, 0; September: 815, 0; March, 1940: 525, 9.2; April: 576, 21.9; May: 611, 34.8; June: 1570, 6.1; July: 2640, 0.01; August: 2000, 0; September: 1250, 0. Examination of these figures indicates a relatively high snail infection in spring and little or no infection in fall and winter. A similar study of *Talorchestia* indicates little infection with metacercariae in early spring, but an increase to a maximum in late July and August.

These results might be interpreted as follows: When the birds arrive early in February and March during their northward migratory period, eggs of the parasite are dropped in the feces along the beach as the birds feed at low tide. Miracidia hatch, penetrate *Nassa obsoleta,* and develop into sporocysts. The birds stay at Woods Hole only for a few days at this time and then resume their northward trek to the breeding grounds. When they return in the fall, the cercariae have matured, penetrated the beach fleas, and developd into ripe metacercariae. The birds, then, may become reinfected and carry the worms back on their southward migration. Possibly the snails might become reinfected at this time also, the developing larvae remaining dormant through the winter. But since little or no infection was found in early spring, this hypothesis does not seem well grounded.

Discussion and Conclusions

Until the life cycles of *Gynaecotyla nassicola, Maritrema arenaria* (Hadley and Castle, 1940), and *Spelotrema nicolli* (Cable and Hunninen, 1940) were described, an understanding of the relationships of the Microphallidae was handicapped considerably. The genera of this family were included with the Heterophyidae on purely superficial morphological characters. Ward (1901) erected the subfamily Microphallinae to contain these genera, but he still retained them under the Heterophyidae. Travassos (1921) raised the Microphallinae to family rank, excluding it from the Heterophyidae, as did Viana (1924). Witenberg (1929) likewise excluded the Microphallinae from the Heterophyidae. In spite of these observations, various investigators have retained the microphallids under the Heterophyidae. It seems pertinent at this point, therefore, to indicate briefly the main differences between these two families of trematodes. In the Microphallidae, the uterus lies either wholly posterior to or overlapping the testes; there is a well-developed acetabulum, not enclosed with the genital suckers; the genital pore is not closely associated with the acetabulum; the excretory system is always of the formula $2[(2+2)+(2+2)]$; and the life cycle always includes a crustacean as the second intermediate host. In the Heterophyidae, the uterus lies anterior to the testes; the acetabulum is usually closely associated with the genital structures; the excretory system is never of the above pattern, but consists of large numbers of flame cells on each side of the body; and the life cycle usually includes a fish as the second intermediate host. In the first family, a crustacean-eating animal (usually a bird) is the definitive host, whereas in the second family a fish-eating animal (mammal or bird) is the definitive host. These characters, along with other morphological features, render impossible the inclusion of members of one with those of the other. As pointed out by the writer (1939*c*) and Cable and Hunninen (1940), the complete life cycle of a species should be known before final conclusions can be drawn with respect to relationships, distribution, etc.

The infection of second intermediate hosts through the respiratory system by both *Gynaecotyla nassicola* and *Spelotrema nicolli* would indicate that other members of this family infect the hosts through this system rather than through the digestive tract. Depending on the type of respiratory apparatus of the particular crustacean, the cercariae are either swept in passively through a gill chamber and then penetrate, or actively bore into branchial lamellae.

Considering the results obtained from a seasonal-distribution study of the northward range of the cycle, it would prove of considerable in-

terest to examine the different hosts at the southern range. A similar distribution might occur. If this were so, then added evidence for the hypothesis that the northern snails do not remain infected during the winter would be obtained.

A specificity for hosts is evidenced to a marked degree by the different stages of *G. nassicola*. *Nassa obsoleta* and *Talorchestia longicornis* are the only intermediate hosts found infected or infectable, of the many examined. The distribution of these hosts in the vicinity of the definitive host may account for this phenomenon. Should the habits of other mollusks and crustaceans bring them in proximity to the birds, then they in turn might be expected to become infected. Yet, since no other species could be experimentally infected, the ecological factor is not the determining one. Some physiological difference seems evident. Host preference has been reported by various writers (Lühe, 1909; Dubois, 1929; Wesenberg-Lund, 1934; Rankin, 1939*c*; etc.) and seems to be a fairly common phenomenon.

Summary

The life cycle of the microphallid trematode, *Gynaecotyla nassicola* (Cable and Hunninen, 1938) Yamaguti, 1939, has been determined experimentally and the various stages described and figured. The miricidia penetrate the mud snail, *Nassa obsoleta* (Say), develop into oval sporocysts that produce daughter sporocysts and cercariae of the ubiquita type. The cercariae penetrate the branchial lamellae of the sand flea, *Talorchestia longicornis* (Say), then migrate through the tissues to the pericardial cavity where they encyst. Metacercariae develop into adults when the crustaceans are eaten by shore birds (plovers, sandpipers, etc.) or fed to experimental hosts (gulls).

The ecology of the hosts and parasite is discussed, indicating the close relationship between the distribution of the various animals involved. The differences between the Heterophyidae and the Microphallidae are indicated.

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NITROGEN CONTENT AND DISTRIBUTION IN EGGS OF MELANOPLUS DIFFERENTIALIS DURING EMBRYONIC DEVELOPMENT [1]

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Introduction

During the past few years, attention has been given to studies on the physiology of the egg and embryo of the grasshopper, *Melanoplus differentialis*. Since most of this work has been based on the intact egg as a unit, a growth curve based on the total nitrogen as an index of the protein content seems desirable as a further basis of reference. A study has therefore been made to determine the nitrogen content of eggs of various ages and also the distribution of this nitrogen among shell, yolk and embryo.

Materials and Methods

The preparation of the eggs consisted in removing them from the pods in which they were laid and washing in distilled water to remove any adhering substances. They were kept at 25° C. until they had reached the desired stage or until they had entered the diapause, or blocked state (Slifer, 1932). In order to obtain postdiapause eggs of a known developmental stage, the eggs were subjected to cold (5° C.) soon after diapause began and kept at that temperature for several months. When the temperature is again raised to 25° C., they resume development and hatch in 18 or 19 days. Before analysis, samples of the eggs were examined after dissection to make sure that all were in the desired developmental stage.

For the first part of the study, the original Kjeldahl method for total nitrogen determination as outlined in Hawk and Bergeim's "Practical Physiological Chemistry" (but using a smaller apparatus) was used on lots of about 50 diapause eggs. Some of the lots contained eggs from one pod only, while others were obtained by mixing eggs from 3, 4, or 5 pods. These mixtures gave a value nearer the average, as the eggs of one pod tend to vary less widely than eggs from different pods.

The reagents used were a digestion mixture of 3 grams Cu_2SO_4 and

[1] Aided by a grant from the Rockefeller Foundation for work on the physiology of the normal cell.

1 gram K_2SO_4 in 300 cc. concentrated H_2SO_4; concentrated NaOH solution, for neutralization; 0.0706 N HCl (1 cc. $\equiv$ .9886 mg. N), for receiving the NH_3; and 0.1074 N NaOH, for back titration. The amounts used were about 15 cc. of the digestion fluid, sufficient NaOH to provide an excess of alkali after neutralization of the acid, and approximately 10 cc. of HCl (added from a stopcock burette). Back titration was done with a 50 cc. Mohr burette and with phenolphthalein as an indicator.

The larger part of the work was done by means of Keys' modification of the Kjeldahl technique for rapid microanalysis (Keys, 1940). With this modification, quantities of 0.01–20.0 mg. of total nitrogen per sample may be analyzed. The digestion was carried out by a mixture of Cu_2SO_4, K_2SO_4, and H_2SO_4 as in the previous Kjeldahl method and phenolphthalein was used as an indicator. These are not the reagents used by Keys, but the change does not appreciably affect the accuracy of the method. The HCl was measured into the receiving tube from a .2 cc. pipette graduated in hundredths and .2, .3, or .4 cc. of the acid were used depending upon the sample to be analyzed. The acid was the same as in the previous method (1 cc. $\equiv$ .9886 mg. N) but the NaOH was 0.10025 N. One to 5 cc. of the digestion mixture were required. Titration was done by means of a burette made from a similar .2 cc. pipette fixed with a 1 cc. syringe according to the principle of the Linderstrøm-Lang pipette (Glick, 1935). It can be easily controlled and read to one thousandths of a cc.

Single eggs were analyzed and in addition determinations were run on embryos, shell and yolk separately. The embryos were dissected out and washed, the shells washed, and the washings and yolk combined. Each part was then analyzed separately. For the earlier stages 2 embryos were analyzed since one contains too little nitrogen for accurate determination. A complete series of stages both before and after diapause were analyzed by this procedure. Until the seventh day after laying, the embryos are too small to handle, so no determinations were possible on these younger stages. In late postdiapause eggs, after the yolk mass was completely engulfed by the embryo (about 7 days postdiapause), the unabsorbed yolk was pressed out of the embryo and analyzed separately although some workers include it with the embryo. In all cases, 1-cc. samples of a known solution (1 mg. NH_4Cl in 10 cc.) were analyzed after every 3 to 5 determinations as a check on the reagents and apparatus. If the error exceeded 2 per cent, the determinations done just previously were discarded.

Accuracy

Macrodeterminations were used only when the results on known samples were within 5 per cent of the known value. This error is rather large, but even using 50 eggs the quantity of nitrogen is close to the lower

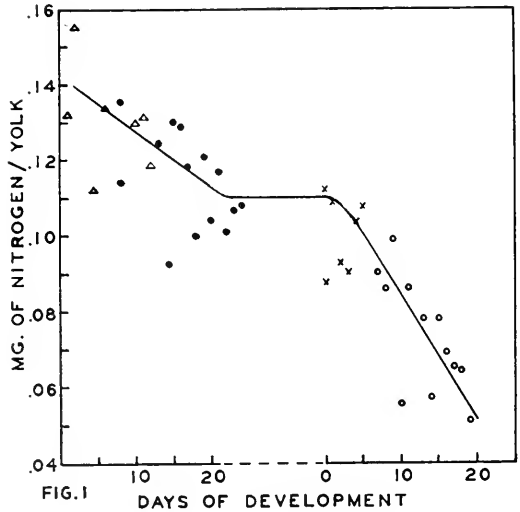

FIG. 1

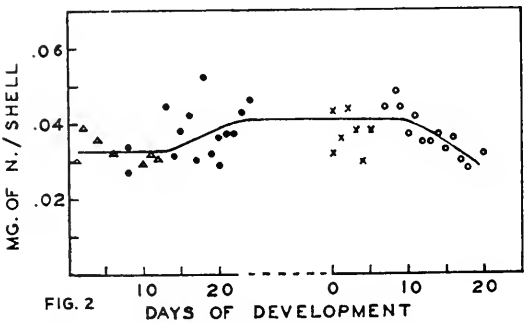

FIG. 2

Fig. 1. Yolk nitrogen. Each point indicates one determination and each pod is represented by a different symbol. The diapause period, which lasts for several months, is shown by the dotted portion of the abscissa. The level of the curve during this period was determined from an average of several analyses.

Fig. 2. Shell nitrogen. The symbols are the same as in Fig. 1.

limit for accurate determination by this method, and too many eggs would have been required to obtain greater accuracy. However, variations between lots from different pods were as much as 20 per cent so that the error in determination is not too great for statistical study.

All determinations of known solutions done by microanalysis were

within 2 per cent of the theoretical value or the experimental analyses just preceding were discarded. The control values ranged between 101.8 per cent and 98.1 per cent but 99.8 per cent and 100.8 per cent were the values more often found. These values correspond to burette readings of 69 and 70 mm.[3] while the extremes used were 68 and 71. The 2 per cent accuracy is all that is necessary since individual variation may be as much as 20 per cent between diapause eggs from different pods.

Results

Macrodeterminations were used to establish the value for the total nitrogen of the intact diapause egg. The eggs are quite variable and therefore a wide range of nitrogen values even for eggs from the same pod exists. The variation is even greater when different pods are used. For this reason, it was thought advisable to use the results of the macro-

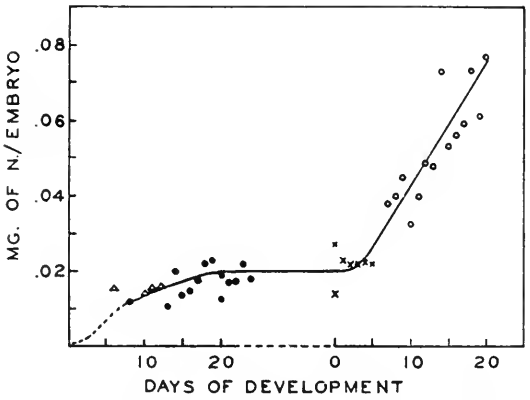

Fig. 3. Embryo nitrogen. Symbols as in the preceding graphs.

analyses to determine the statistical average. The value assigned (174 γ) for the diapause is the average of 18 determinations of 50 eggs each. This serves as a check on the other curves made from micro-determinations on fewer pods.

It will be seen from the yolk curve (Fig. 1) that a wide variation in the yolk nitrogen accounts for most of the variation of the egg as a whole. This can be seen by comparing the individual points on the graph of the yolk (Fig. 1) with those on the curve plotted by adding together the 3 parts of the egg (Fig. 4). In spite of the rather wide variation, a definite trend downward may be seen. The yolk nitrogen drops from about 80 per cent of the total nitrogen at laying to about 60 per cent at diapause. During diapause it remains constant and when development is resumed it decreases rapidly from 110 γ at diapause to 50 γ at hatching.

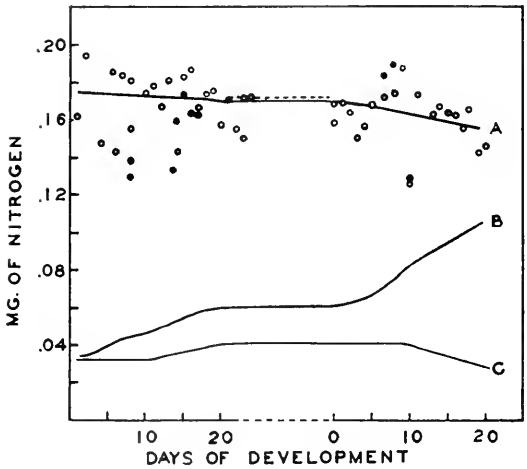

FIG. 4. A composite of the three preceding graphs No individual determinations were used but the three smoothed curves were added. *C*, shell *N*; *B*, shell and embryo *N*; *A*, shell plus embryo plus yolk *N*. The ordinate length beneath curve *C* is a representation of the shell nitrogen; that between *B* and *C*, the embryo nitrogen; and between *A* and *B*, the yolk nitrogen. The level of the broken line is the level for diapause determined by the macromethod.

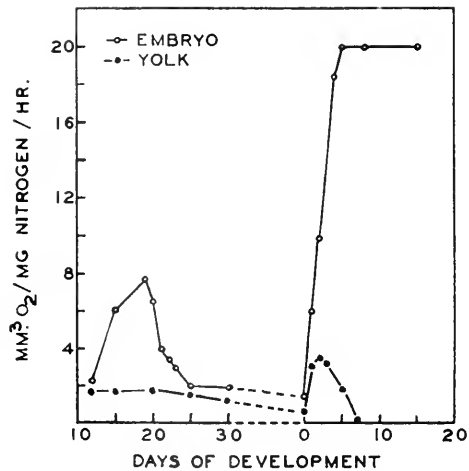

FIG. 5. Curves obtained by dividing the O_2 consumption curves of Boell and Bodine (1936) by the embryo and yolk *N* curves of this paper, showing the changes in basal respiration, i.e., mm.^{3} O_2/mg. nitrogen/hr., during embryonic development.

The shell nitrogen (Fig. 2) was found to be about 30 γ per egg until about the tenth day. Between the tenth and twentieth days it rose to more than 40 γ per egg, which was maintained during the diapause period. Between 5 days postdiapause and hatching, the shell nitrogen decreased to about 35 γ.

The curve for embryo nitrogen (Fig. 3) rises steadily during prediapause development, is level at diapause, and again rises rapidly in the postdiapause period.

Figure 4 is a composite of the three preceding graphs. The points plotted for the nitrogen of the whole egg were found by analyzing whole eggs (solid symbols) and by adding the values found for embryo, shell, and yolk of the same eggs (open symbols). These points are extremely scattered and no curve can be drawn accurately based on these points. The curve drawn was made by adding the smoothed curves of the separate parts. The diapause level thus determined is not significantly different from the level taken from the average of the macrodeterminations on diapause eggs.

Discussion

The curve (Fig. 4) for the nitrogen of the whole egg is too indeterminate for any accurate quantitative analyses of the nitrogen behavior but it can be seen by the addition curve that no significant change, either increase or decrease, in nitrogen during the embryonic period occurs. The organism seems incapable of utilizing atmospheric nitrogen.

The early rise in shell nitrogen, as well as the drop in yolk nitrogen, corresponds to the formation of the white cuticle which begins at about the tenth day (Slifer, 1937). The loss of nitrogen from the shell in the postdiapause period is doubtless due to the digestion of this layer preparatory to hatching (Slifer, 1937).

The embryo nitrogen curve is comparable in shape to curves for various physiological activities of the embryo. A mm.^{3} O_2/mg. embryonic N/hr. curve has been made (Fig. 5) by dividing the mm.^{3}/embryo/hr. curve of Bodine and Boell (1936) by the mg. N/embryo curve of this paper, which may be used as a basal curve for future work. The rate of O_2 consumption per mg. of embryo nitrogen rises steadily in prediapause, but drops rapidly at the onset of diapause, the diapause level being 1.7 mm.^{3} O_2/mg. N. As soon as development resumes the rate rises again, until, on the fifth day, it reaches 20 mm.^{3} O_2/mg. N where it remains practically constant throughout the remainder of the embryonic period.

During prediapause the O_2 consumption of the yolk (Fig. 5) is a constant value, 1.8 mm.^{3} O_2/mg. N. It drops during diapause to .5

mm.^{3} In the postdiapause period, it rises to a peak of 3.7 mm.^{3} in 2 days and then drops rapidly to zero when the yolk has been engulfed by the embryo.

Summary

The total nitrogen content of the egg of the grasshopper, *Melanoplus differentialis,* has been determined and also the changes in its distribution among shell, embryo, and yolk. The nitrogen content of the whole egg is constant while the embryo nitrogen increases at the expense of the yolk nitrogen. The changes in shell nitrogen correspond to the formation and digestion of the cuticle. No change occurs during diapause.

A basal metabolism curve is computed from the embryo nitrogen curve of this paper and the oxygen consumption curve of Boell and Bodine (1936) which shows that the oxygen consumption rate rises to 20 mm.^{3} O_2/mg. N at 5 days postdiapause and remains steady at that value. A similar curve is computed for the yolk. The metabolic rate is constant in the prediapause at a value of 1.8 mm.^{3} O_2/mg. N and in the postdiapause drops from a peak at 2 days to 0 at 10 days. Both curves show very low rates during diapause.

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FACTORS DETERMINING THE DISTRIBUTION OF POPULATIONS OF CHAETOGNATHS IN THE GULF OF MAINE[1]

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In aquatic environments, and particularly in large bodies of water, the movement of the water itself may profoundly affect both the distribution and numbers of the population. Pelagic organisms are carried with the water in its drift through each given region. Numerical abundance may thus depend on conditions in external regions from which the water comes. The moving water affects the climate of the region into which it flows, for it carries with it its inherent temperature, salinity, etc. At the same time these properties of the water may be profoundly altered in its course, through interaction with the atmosphere and with other bodies of water. The maintenance of a population in a given region under these conditions depends upon a balance of dynamic factors; the drift of the water and its interaction with its environment, as well as upon the rate of reproduction and mortality of the population under the environmental conditions determined by these circumstances (Russell, 1936). The pelagic population is at the mercy of the hydrodynamic factors, since it must move with the water. It is only by taking advantage of fortuitous hydrographic conditions, such as the existence of permanent eddies and dead waters, that they may gain a truly endemic relation to a given region.

The use of plankton as indicators of water movements has attracted deserved attention of late (Russell, 1936*a*, 1939). The effect of the circulation of water on the distribution and maintenance of permanent populations of specific organisms has received less explicit attention, but is exemplified by the studies of Damas (1905), Sømme (1933, 1934) and Redfield (1939).

As Huntsman (1919) has shown, the chaetognaths are an unusually interesting group for studies in oceanic zoögeography. Of the half-dozen species found in the coastal waters between Cape Cod and the Grand Banks, three are of deep-water origin and are drawn into the deeper basins of the Gulf as an immigrant population which perishes without reproducing. Three other species are inhabitants of the upper layers of the sea, of which one breeds endemically on the continental

[1] Contribution No. 279 from the Woods Hole Oceanographic Institution.

shelf, while the other two are immigrants from the oceanic waters offshore. These six species must have essentially similar habits of life, yet each has a characteristically different distribution, depending upon the water body in which it maintains itself endemically, and upon the degree to which these waters are drawn into the various regions of the coast. The value of Sagittae as indicators of water movements in British waters has been demonstrated by Russell (1935, 1936*b*), Fraser (1937, 1939) and Pierce and Orton (1939).

The present paper is an examination of the populations of chaetognaths found in the Gulf of Maine during the year 1933–34, when the research vessel of the Woods Hole Oceanographic Institution, the "Atlantis," made a systematic survey of the region. While the results confirm in the main the observations of Bigelow (1926) in this region and

TABLE I

Statistics of collections of chaetognaths in the Gulf of Maine, 1933–1934.

Dates of Cruise	Number of Stations at Which Chaetognaths were Present						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total Stations
Sept. 2–14, 1933	30	12	6	2	1	14	35
Dec. 2–11, 1933	17	9	5	1	0	13	22
Jan. 3–13, 1934	11	7	2	2	0	9	12
Mar. 21–28, 1934	10	8	0	0	0	15	18
April 17–May 13	20	9	2	0	0	16	32
May 21–June 2	32	20	4	1	0	21	44
June 25–July 1	6	2	0	0	0	2	7
Sept. 17–24, 1934	20	16	4	3	1	15	25
Total	146	83	23	9	2	105	195
Per cent present	75	43	12	46	1	54	

Dates of Cruise	Numbers of Chaetognaths Taken at These Stations						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total
Sept. 2–14, 1933	2,961	104	9	4	2	107	
Dec. 2–11, 1933	3,608	45	17	1	0	300	
Jan. 3–13, 1934	194	8	3	3	0	87	
Mar. 21–28, 1934	105	13	0	0	0	95	
April 17–May 13	785	137	8	0	0	274	
May 21–June 2	1,698	104	21	2	0	289	
June 25–July 1	368	33	0	0	0	2	
Sept. 17–24, 1934	453	534	5	3	5	133	
Total	10,170	978	63	13	7	1,287	12,418
Per cent of total	82	7.9	0.51	0.10	0.06	9.5	—

Dates of Cruise	Mean Catch per Haul at Stations at Which the Species was Present						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total
Sept. 2–14, 1933	98.7	8.7	1.3	2.0	2.0	7.6	
Dec. 2–11, 1933	212.0	5.0	3.4	1.0		22.1	
Jan. 3–13, 1934	17.6	1.1	1.5	1.5		9.7	
Mar. 21–28, 1934	10.5	1.6	—	—		6.3	
April 17–May 13	39.1	15.2	4.0	—		17.0	
May 21–June 2	53.0	5.2	5.3	2.0		13.7	
June 25–July 1	61.5	16.5	—	—		1.0	
Sept. 17–24, 1934	22.7	33.4	1.25	1.0	5.0	8.9	
Mean catch per haul for entire period	69.8	11.8	2.74	1.44	3.5	11.3	

Dates of Cruise	Per Cent of Total Hauls in Each Period at Which Each Species was Present						
	S. elegans	*S. serratodentata*	*S. maxima*	*S. lyra*	*S. enflata*	*E. hamata*	Total
Sept. 2–14, 1933	86	34	17	5.7	2.9	40	
Dec. 2–11, 1933	77	41	23	4.5		59	
Jan. 3–13, 1934	92	58	17	17		75	
Mar. 21–28, 1934	56	45	0	0		83	
April 17–May 13	63	28	6.3	0		50	
May 21–June 2	73	45	9	2.3		48	
June 25–July 1	86	29	0	0		14	
Sept. 17–24, 1934	80	64	16	12	16	60	
Entire period	75	43	12	4.6	1	54	

of Huntsman (1919) in Canadian waters, it has seemed worthwhile to examine them together with those of Bigelow and Huntsman from the point of view outlined above.

Material and Data

General Statistics

The cruises were planned to include lines of stations extending from shoal water across the Gulf so as to sample all the principal parts of the Gulf and the enclosing banks. While exigencies of weather and the loss of nets interfered with the completeness of the program, the 195 hauls included in this study amount to a fairly satisfactory random sampling of the region, throughout the year.

The collections were made with a standard 1.5 meter Heligoland larva net (No. 0 silk, 38 meshes to the inch) drawn vertically from a point near the bottom to the surface. The opening of the net had an area of 1.7 square meters. Table I contains some statistical data on the collections.

Numerical Abundance.—The 12,418 specimens taken give a mean catch of 64 per haul or 37.5 per square meter of sea surface. Of these by far the most abundant was *S. elegans,* amounting to 82 per cent of the total. With *S. serratodentata* yielding 7.9 per cent, these two shallow-water forms accounted for 90 per cent of the total population. Of the three deep-water species *E. hamata* accounted for 9.5 per cent of the catch, with *S. maxima* and *S. lyra* making up 0.5 and 0.1 per cent respectively.

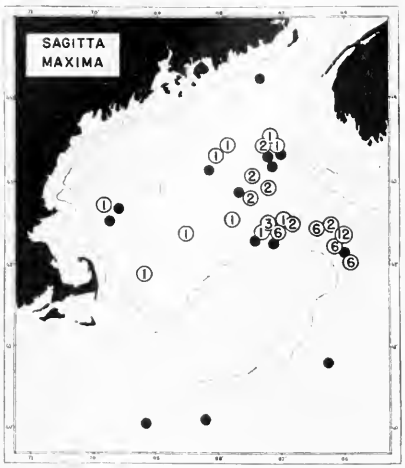

FIG. 1

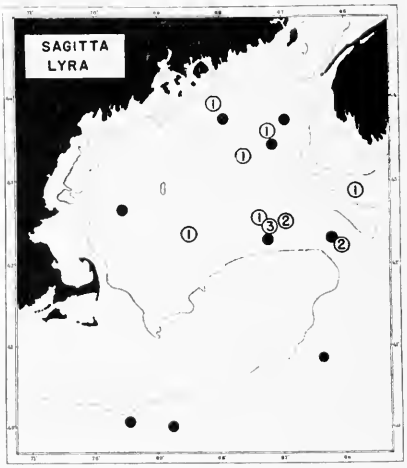

FIG. 2

FIG. 1. Locations at which *Sagitta maxima* has been taken in the Gulf of Maine.

FIG. 2. Locations at which *Sagitta lyra* has been taken in Gulf of Maine. Figures in circles indicate the numbers taken per haul from "Atlantis" in 1933–34. Solid circles indicate Bigelow's captures.

The relative breadth of distribution of the species is indicated by the percentage of stations at which each was taken. The general order is the same as for the numerical abundance. The species differ, however, far less in distribution than in numerical abundance, e.g., *S. serratodentata* and *E. hamata* which accounted for only 8 and 9 per cent of the total numbers but occurred at more than 40 and 50 per cent of the stations.

Numerical abundance depends on both the extent of distribution and the density of population within that distribution. A general idea of the latter is given by the mean size of the catch at each station at which a given species is present. Again the order is essentially the same, *S. elegans* leading with a mean density of 70 per haul, *S. serratodentata* and *E. hamata* following with 12 and 11 per haul, while *S. maxima* and *S. lyra* occur sparsely when at all.

Seasonal Fluctuations

Sagitta elegans and *S. serratodentata* show a decline in total numbers taken, and in the mean density of the population at the stations where they were present during the colder part of the year. The figures are irregular as both forms occur occasionally in dense swarms, but it appears that these species are reduced to perhaps one-tenth their numbers during the winter. The cruises in December, January and March did not sample adequately the waters of Georges Bank where the larger catches of *S. elegans* were taken at other seasons and this may exaggerate the apparent effect of winter on this species, although it can scarcely do so in the case of *S. serratodentata,* which occurs chiefly over the deeper part of the basin which was adequately sampled.

Contrasted with these inhabitants of the upper levels, in which the seasonal change in temperature is great, the deep-water species *E. hamata* shows no systematic fluctuation either in total numbers, the percentage of stations at which it is present, or numbers taken per haul, during the year. Bigelow (1926) has pointed out that this is to be expected since the depths at which it lives are not subject to much change in temperature.[2]

The Distribution of the Deep-Water Species

Sagitta maxima, S. lyra and *Eukrohnia hamata* are inhabitants of the deeper waters of the Gulf of Maine. Figures 1 and 2 record the location and numbers of the two former species taken in each haul while Fig. 3 shows the general pattern of distribution of the more abundant *E. hamata.* All our specimens of *S. maxima* were taken in water of over 170 meters depth and it occurred in greatest abundance in depths of over 200 meters. It seems safe to conclude with Huntsman and Bigelow that it occurs only in the deeper parts of the Gulf, chiefly below the 150-meter level. The distribution of the catches of *S. lyra* is essentially the same as that of *S. maxima.* The regional occurrence of *E. hamata* is similar to that of the deep-water sagittae, though it was taken more frequently in the shoaler regions along the margins of the Gulf, occasionally in water of as little as 100 meters depth. It appears to occur rather nearer the surface than do the deep-water sagittae.

The distribution of catches of the three species agree in showing the highest frequency of successful catches and the greatest numbers per haul in the Eastern Channel through which deep water has access to the Gulf. Inside the Gulf the populations are concentrated in the eastern

[2] Bigelow considered that *S. maxima* occurred in the Gulf more frequently in winter, *S. lyra* in summer. Our data do not confirm this. Their numerical abundance is rather closely correlated throughout the year and neither shows a clear seasonal variation.

basin, and diminish in abundance northward and westward as the Eastern Channel becomes remote.

Dynamic contours give the best available information concerning the probable circulation of the deeper water. Contours representing the circulation at 140 meters in May–June, 1934 are indicated in Fig. 5.[3] They represent a pattern which occurs with some variation in detail from month to month at this and greater depths. The center of abundance of the three deep-water chaetognaths lies in the rapidly moving inflow or in the eastern side of the eddy which has its vortex in the deep basin northwest of the Eastern Channel. Once the creatures pass beyond the vortex they become scattered and decrease in numbers.

The distribution of the deep-water chaetognaths suggests strongly that they are carried into the Gulf from offshore, along with the warm

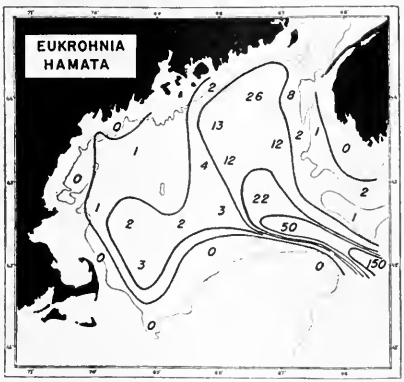

Fig. 3

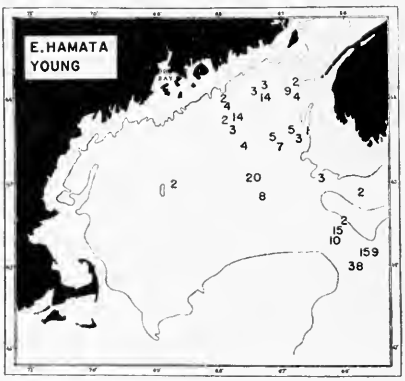

Fig. 4

Fig. 3. Relative abundance of *Eukrohnia hamata* in different parts of the Gulf of Maine. Numbers represent the average catch per haul in various regions.

Fig. 4. Locations at which young specimens of *Eukrohnia hamata* were taken in 1933–34. Numbers indicate the number of young taken per haul at each position.

saline water which Bigelow (1927) found to enter through the Eastern Channel. Since there is no evidence that they breed within the Gulf, they are to be considered as terminal immigrants from other regions in which they breed endemically. While some of those which are carried into the Gulf may complete the circuit of the eddy and be carried out again, the greater number are probably trapped within the dead waters of the inner Gulf, live as long as circumstances permit and die without leaving progeny.

The foregoing observations and conclusions are in entire agreement with those of Bigelow (1926). In certain details, however, our findings

[3] A hydrodynamic analysis of the circulation of the Gulf of Maine during the period of our survey is being prepared by Dr. E. E. Watson, to whom I am indebted for the use of this and subsequent figures.

differ from his. While he considered *E. hamata* to occur with regularity throughout the year, the sagittae were thought to fluctuate with the season, *S. maxima* being taken predominantly in the colder part of the year and *S. lyra* in the warmer. He proposed that *S. maxima,* an inhabitant of northern waters, is barred from the Gulf by the warmth of the water entering the Eastern Channel during the summer months. The statistics recorded in Table I fail to show a clear fluctuation in any of the deep-water species which may be correlated with the season. That *S. maxima* may enter the Gulf at a time when the water is warmest is evidenced by our taking 12 and 6 specimens in two hauls made in the Eastern Channel in May when the temperature of the deeper water was 8°–9° C. and 2 in a September haul from water varying in temperature from 6.5° to 8.76°.

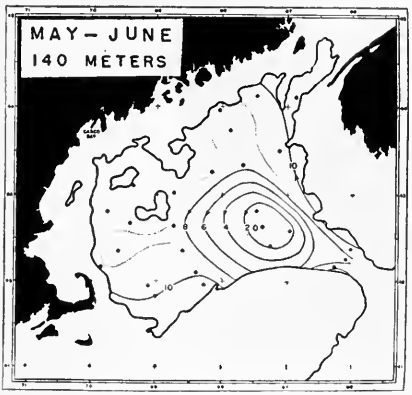

FIG. 5

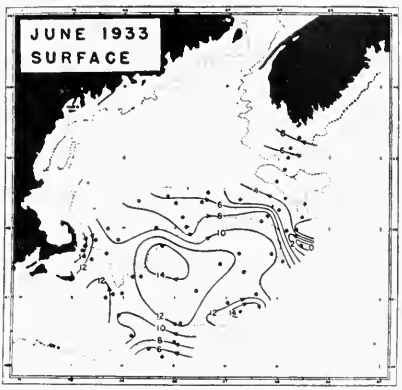

FIG. 6

FIG. 5. Dynamic contours indicating theoretical circulation in the basin of the Gulf of Maine at depth of 140 meters in May–June, 1934.

FIG. 6. Dynamic contours indicating theoretical circulation at surface of Georges Bank in June, 1933.

We believe he was misled, particularly in the case of *S. lyra,* of which he took only 6 examples, by the fact that his cruises did not sample the center of abundance of the populations as thoroughly as the more remote and colder periphery of the range.

Factors Limiting the Distribution within the Gulf

In attempting to evaluate the relative importance of the circulation of the water and of environmental factors in determining the abundance and distribution of the species, it is crucial to inquire whether the numerical relations are due to the degree to which the three species are "adapted" to the conditions in the Gulf, or whether they are to be explained by the relative numbers in which they enter from offshore.

In the former case the numerical ratios should shift as one compares waters increasingly remote from the point of entrance. In the latter they should remain unchanged.

To examine these possibilities, we have divided the catches in which any of the species occurred into three groups: (1) those taken in the deep water of the Eastern Channel (east of 66°30′ and south of 42° 40′N); (2) those lying immediately within the basin (a quadrangle lying between 42°10′ and 43°30′N and 66°30′ and 67°30′W), and (3)

TABLE II

Relative numbers of deep-water chaetognaths taken in different areas in Gulf of Maine.

Total Numbers	*E. hamata*	*S. maxima*	*S. lyra*
Eastern Channel	434	34	2
Southeastern quadrangle of basin	411	25	6
Remainder of basin	442	4	5
Total area	1287	63	13
Relative Numbers Taken			
Eastern Channel	100	7.8	0.5
Southeastern quadrangle of basin	100	6.1	1.5
Remainder of basin	100	0.9	1.1
Total area	100	5.0	1.0
Stations Present			
Eastern Channel	9	6	1
Southeastern quadrangle of basin	19	11	3
Remainder of basin	87	5	5
Total area	105	23	9
Relative Numbers of Stations Present			
Eastern Channel	100	67	11
Southeastern quadrangle of basin	100	58	5.7
Remainder of basin	100	5.7	5.7
Total area	100	22	8.6

the remainder of the Gulf. These three areas yielded *E. hamata* in about equal numbers. They differ in proximity to the external sources of the deep water of the Gulf.

The numbers of specimens and the relative numbers of each species taken in these areas are given in Table II. They show that a great disparity between the abundance of the three species already occurs in the water entering the Channel. In the main the relative abundance is established by conditions external to the Gulf, and cannot be attributed merely to the selective effect of conditions within.

There is, however, clear evidence that some selective mechanism is at work limiting the penetration of *S. maxima* into the remoter region. The ratio in which *S. maxima* is taken in proportion to the catch of *E. hamata* is eight times as great in the Eastern Channel as in the remoter region. The distribution of *S. lyra* appears to resemble that of *E. hamata* more closely than that of *S. maxima*.

TABLE III

Catches of Sagitta maxima and Sagitta lyra classified according to highest temperature of the water column (below 100 meters) from which they were taken.

Temperature, ° C.	*S. maxima*							
	5–5.5	5.5–6.0	6.0–6.5	6.5–7.0	7.0–7.5	7.5–8.0	8.0–8.5	8.5–9.0
Number per haul	1	1	2	3	2	6	12	6
		1			2	2	6	2
					1	1	6	
					1		3	
					1		2	
							1	
Total	1	2	2	3	7	9	30	8
Per cent of total	1.59	3.18	3.18	4.76	12.7	14.3	47.7	12.7
Integrated per cent	1.59	4.77	7.95	12.71	25.4	39.7	87.4	100
Mean catch per haul	1	1	2	1.5	1.3	3	5	4

Temperature, ° C.	*S. lyra*							
	5–5.5	5.5–6.0	6.0–6.5	6.5–7.0	7.0–7.5	7.5–8.0	8.0–8.5	8.5–9.0
Number per haul					1	1	3	
					1		2	
					1		2	
					1		1	
Total	0	0	0	0	4	1	8	
Per cent of total	0	0	0	0	30.8	7.7	61.6	
Integrated per cent	0	0	0	0	30.8	38.5	100	
Mean catch per haul	0	0	0	0	1	1	2	

In the discussion of the geographical distribution of the chaetognaths,—as of marine plankton in general,—temperature and salinity are commonly considered as factors limiting the distribution of the species. The water in which the chaetognaths of the depths appear to be carried as they penetrate the Gulf undergoes admixture with subsurface layers. As a result it becomes colder and less saline. While not wishing to suggest that these factors are not in a broad way influential in determining the survival of the species, we do wish to question whether they, or

similar environmental conditions, are essential in accounting for the differences in their distribution within the Gulf of Maine. While our data suggest that *E. hamata* invades colder water more frequently than *S. maxima* and *S. lyra,* there is no evidence that these latter cannot survive in water at least as cold as any in which we have taken the former species. Bigelow (1926, p. 325) records captures of *S. maxima* off the continental slope in waters of 3° to 6° C. and considers this species to be a distinctly cold-water form. *S. lyra* was taken by him in water of 6° C.—below which only 8 per cent of the total catch of *E. hamata* was taken. Though it may be considered a creature of relatively warm water, we have shown that it penetrates into the colder parts of the Gulf, relatively as frequently as does *E. hamata.*[4]

We believe the disparity in distribution of the deep-water chaetognaths in the Gulf can be explained more readily in another manner. We have presented in Tables III and IV data showing the incidence of

TABLE IV

Catches of Eukrohnia hamata classified according to highest temperature of the water column (below 100 meters) from which they have been taken. Total catch = 1287.

Temperature, ° C.	2–3	3–4	4–5	5–6	6–7	7–8	8–9	9–10	10–11
Total hauls	7	13	15	32	40	28	11	4	1
Total caught	7	11	33	61	213	407	310	244	1
Per cent of total	0.54	0.86	2.56	4.74	16.5	31.5	24.0	18.9	—
Integrated per cent	0.54	1.40	3.96	8.70	25.2	56.7	80.7	99.0	100
Mean catch per haul	1.0	0.85	2.2	1.9	5.3	14.5	28.2	60	1

the species in water of different maximal temperatures. The catches have been classified according to the temperature of the water in which they were taken. We have chosen the highest temperature recorded below 100 meters, since there is no way of knowing the level at which an animal caught in a vertical haul is taken, and since this temperature will represent the least departure from the condition under which the water entered the Gulf. The temperature profiles shown in Fig. 7 give

[4] Salinity cannot readily be separated from temperature as a possible limiting factor within the deep water of the Gulf because variations in temperature and salinity alike are due to mixtures in varying proportion of the deep, warm, and saline water with the superficial, cold, and dilute water. As a result, the temperature-salinity diagram for waters in all parts of the Gulf below the level of homogeneous winter mixing is almost a straight line and is nearly the same in each region.

It is, moreover, extremely difficult in view of what is known experimentally concerning the osmotic regulation of marine invertebrates, to believe that changes of one or two parts per mille would vitally affect animals native in 34 or 35 ‰ salinity.

some idea of the possible range of temperature in various regions of the Gulf.

Tables III and IV show the result of this classification. Though not corrected for the relative number of hauls taken in each kind of water, this correction may be neglected in comparing the different species since the data for each are derived from the same collection of catches. For each species the numbers fall off sharply as the temperature declines. In the case of *S. maxima* and *S. lyra* the great change occurs at about 7° C. *E. hamata* appears to succeed better in penetrating the colder parts of the Gulf, the numbers caught falling markedly only below 6° C.

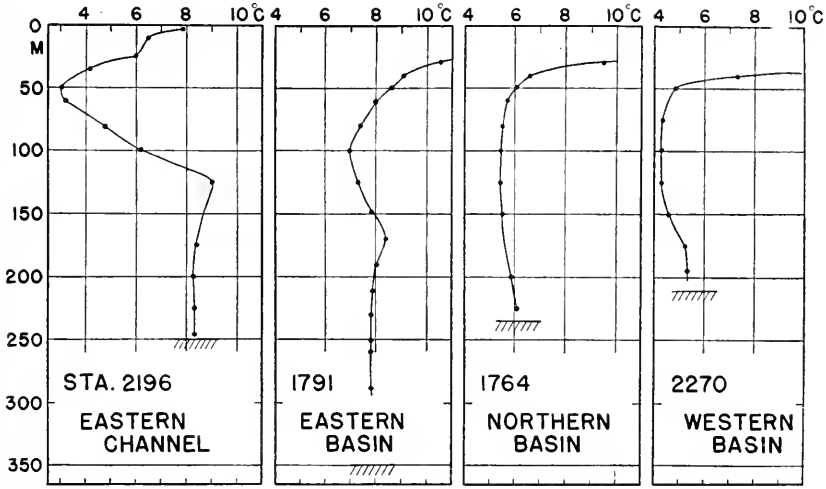

FIG. 7. Distribution of temperature with depth at a number of stations at which *Sagitta maxima* was taken.

In Fig. 8 we have plotted the integrated data showing the fraction of the total catch of each species which has penetrated to regions of decreasing temperature. The curves in Fig. 8 might be interpreted as representing the relative tolerance of individuals of the three species for the physical characteristics of the water. When the dynamic nature of the situation is considered, we believe they may be more plausibly interpreted as depending on the time of survival to be expected by the numbers which enter the Gulf, and irrespective of any change in the physical characters of the environment to be encountered therein except insofar as the conditions in the Gulf are unsuitable for its reproduction. Since the changes in the physical characteristics of the deep water depend on mixing processes, and since these must proceed relatively uniformly in all parts, the change in temperature and salinity is a function of the

time during which the water has been exposed to these processes. Temperature and salinity are thus a function of the age of the deep water in the Gulf. The abscissa of Fig. 8 may be replaced by a time scale reading from right to left and measuring in a general way the time since the water with its included population entered the Gulf.

With these considerations in mind, the curves in Fig. 8 may be interpreted as probability integrals representing the expectancy of life of the groups of chaetognaths which appear in the Eastern Channel. *E. hamata* has an expectancy of longer life than the others. Hence it has time to be carried into the remoter parts of the Gulf in large numbers

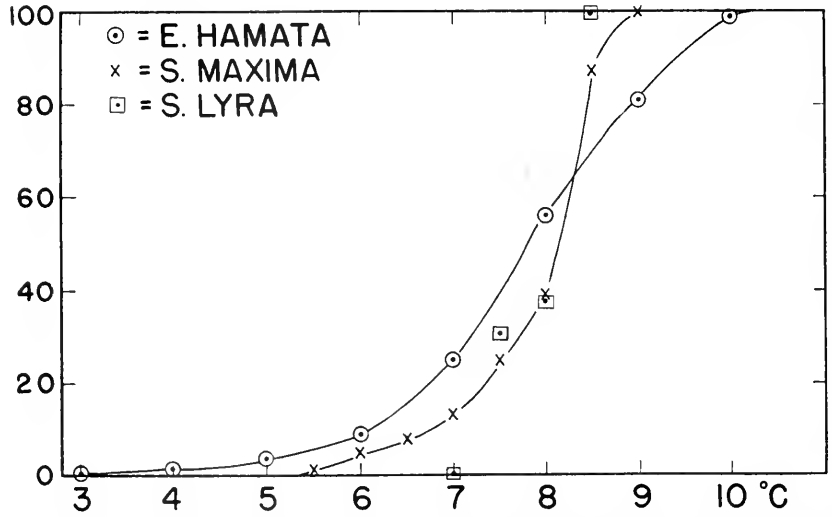

FIG. 8. Percentage of the total catch of *Eukrohnia hamata, Sagitta maxima* and *S. lyra* taken at stations at which the water below 100 meters was colder than the temperature shown on the abscissa. Based on integrated percentages shown in Tables II and III.

before it dies. Why its expectancy is longer may depend merely on its inherent longevity. It may, however, depend on its relative age at the time when it enters the Gulf. For the latter alternative there is some evidence, for we have found that young individuals of this species appear in the eastern side of the basin during the winter months (Fig. 4). This interpretation renders any special considerations of the relative fitness of the environment for the three species unnecessary, though it does not exclude them as contributory causes.

The distribution within the Gulf appears to depend essentially upon the current system and upon the age of the population which enters the region. This in turn must depend upon the relative remoteness from

the Eastern Channel of the centers of reproduction for each species—a remoteness to be measured in terms of the length and velocity of the transport system which bears the population to its destination.

The Sources of the Deep-water Population

Turning to the external sources of the population which enter the Gulf, the question is: Do the different species have a common source in the slope water which enters the Eastern Channel, or do they come from different sources to be mingled during their passage into the Gulf? The first alternative demands the same relative abundance of the species in the slope water as within the immediate entrance of the Gulf. Huntsman records catches of chaetognaths from 20 stations along the coast of the maritime provinces in water of over 1,000 meters depth made with vertical hauls from depths between 200 or 375 meters and the surface. In these hauls he counted 408 *E. hamata*, 235 *S. maxima*, and 49 *S. lyra*. The numerical ratios are 100:59:12. The relative abundance of *S. maxima* and *S. lyra* are almost the same as their occurrence in the Gulf of Maine. *E. hamata* was, however, very much scarcer relatively in these collections than in those made in the Gulf of Maine and was absent from a number of stations at which the others occurred. If Huntsman's catches may be considered characteristic of the slope water to the eastward of the Eastern Channel, then this body—at least in its upper layers—cannot be considered the sole source of the *E. hamata* population of the Gulf of Maine.

Bigelow considered *S. maxima* to come to the Gulf of Maine with the slope water from the northeast. He suggests that *E. hamata* is confined to waters so deep, except in high latitudes, that it never reaches the Gulf of Maine from the oceanic basin abreast of it, a consideration which seems to limit its origin also to the slope water. The absence of *S. lyra* from Huntsman's inshore and eastern stations, together with its more limited distribution in the north Atlantic, seem to preclude its origin from the boreal sources of the slope water. Since Huntsman's and Bigelow's studies were published, Rossby (1936) has shown that there are important forces at work transporting deep oceanic water into the region of the slope water, as the result of which this body owes its origin to oceanic as well as to boreal sources. Redfield (1936) has discussed the ecological consequences of these facts. The diverse origin of the waters which enter the Gulf of Maine and their inhabitants are thus more clearly understood. Hydrographic observations emphasize the complexity of the water bodies occurring close to the mouth of the

Eastern Channel. These facts seem to favor a diversity in the origin of the chaetognaths which penetrate the Eastern Channel.[5]

The Distribution of the Chaetognaths of the Upper Levels

Sagitta enflata, *S. serratodentata*, and *S. elegans*, all of which are more commonly taken in tows from the upper 100 meters, present much greater contrasts in numbers, distribution, and seasonal fluctuations than do the deep-water chaetognaths. Their occurrence and abundance can be attributed to the circulation of water and its relation to the areas suitable for their reproduction in great detail. On the one hand, *S. elegans* is capable of reproducing in the shallow waters which encircle the Gulf and occurs in numbers and at times which can be attributed to the nature and stability of local hydrographic conditions. On the other hand, *S. serratodentata* is an inhabitant of the warm ocean offshore and is carried into the Gulf as a periodic immigrant at times dependent on a cyclic change in the major circulation of the region, while *S. enflata* represents a rare straggler from (hydrographically) more remote regions which can reach the margins of the Gulf only at such times as the temperature of the water permits its survival for a sufficient period.

Sagitta enflata

S. enflata is a tropical form ranging to 40°N in the surface water of the Gulf Stream. Huntsman found it only at the outermost stations along the continental margin and in considerable numbers only at his more western stations in the offing of the Eastern Channel. He captured one specimen in July over the coastal shelf north of Sabro Bank. It has been taken occasionally over the continental slope south of Cape Cod, Bigelow and Sears (1939) recording a large catch of juveniles close to Martha's Vineyard in 1935. South of Delaware it is not infrequent 30 miles in from the continental slope and south of the Chesapeake it occurs frequently close in to land. The invasion of the coastal waters south of Cape Cod occurs at all seasons but most often in the autumn. We captured *S. enflata* on two occasions only. On September 3, 1933

[5] Bigelow found *E. hamata* to vary markedly in abundance from season to season, a fact which might arise from variations in the inflow and its origin from year to year. His capture of *S. maxima* chiefly in winter and *S. lyra* in summer, as well as our failure to find these species dissociated in time may have their origin in similar fluctuations. Bigelow concluded, from hydrographical evidence, that the inflow through the Eastern Channel is variable. Our data have been searched for correlations between the distribution of the species and that of the hydrographic factors in the region of the Eastern Channel, but we have failed to find any good evidence of a dissociation in the time at which the different species enter or in their presence in separate bodies of water within the neighborhood of the Eastern Channel.

one specimen occurred in a haul made in the South Channel in association with large numbers of salpae. On September 23, 1934 five specimens occurred in a haul taken well within the Eastern Channel.

S. enflata is thus one of the many tropical forms which in small numbers find their way in over the margin of the continental shelf in the latter part of each summer. Our records are the first for the margins of the Gulf itself. In the present connection the species is of interest as the extreme example in a series of forms relating frequency of occurrence to the remoteness of the area of reproduction, and to seasonal fluctuations.

S. serratodentata Krohn

This inhabitant of the upper layers of the warmer parts of all oceans extends farther north than most such, being able to survive, though not to breed, in relatively cold water and consequently it extends farther inshore than do most forms of like origin. Huntsman found *S. serratodentata* at practically all stations off the continental slope as far east as the Newfoundland Banks. It extended into the Laurentian Channel over the deep trough but did not penetrate into the Gulf of St. Lawrence. It occurred over the western half of the Nova Scotian Banks well in toward shore and in numbers increasing westward. Bigelow records its occurrence off the continental shelf to the south of Georges Bank and states that from New York southward it is the prevailing chaetognath right in to the shore in warm summers, though outnumbered by *S. elegans* in cooler seasons, at least over the inner part of the shelf as far south as Delaware Bay. North of Chesapeake Bay, and especially east of New York, it is much less frequent close to the land. Bigelow and Sears (1939) find no seasonal fluctuation in its abundance, either inshore or offshore in the coastal belt west of Cape Cod. They consider it to be maintained by local reproduction within that area.

In the Gulf of Maine Bigelow (1926) found it to penetrate the eastern part of the basin regularly and to extend westward along the New England coast in diminishing numbers as far as Massachusetts Bay. He took it only occasionally in small numbers on Georges Bank. It was absent from the southwestern quarter of the basin at all times and disappeared almost completely from the entire Gulf in the late autumn. Its occurrence was highly seasonal, the invasion of the Gulf beginning in late spring and culminating in September. There is no evidence that it reproduces successfully in the Gulf.

Our collections confirm abundantly Bigelow's general conclusions. The center of abundance is in the eastern half of the Gulf, agreeing well with that of the deep-water chaetognaths, though extending into the

shoaler water over Brown's Bank and the Northern Channel. Access to the Gulf is available over this route from the concentrations Huntsman observed on the western Nova Scotia Banks and from the slope water over the Eastern Channel. The inner and western part of the Gulf is penetrated in smaller numbers and in general along the northern side of

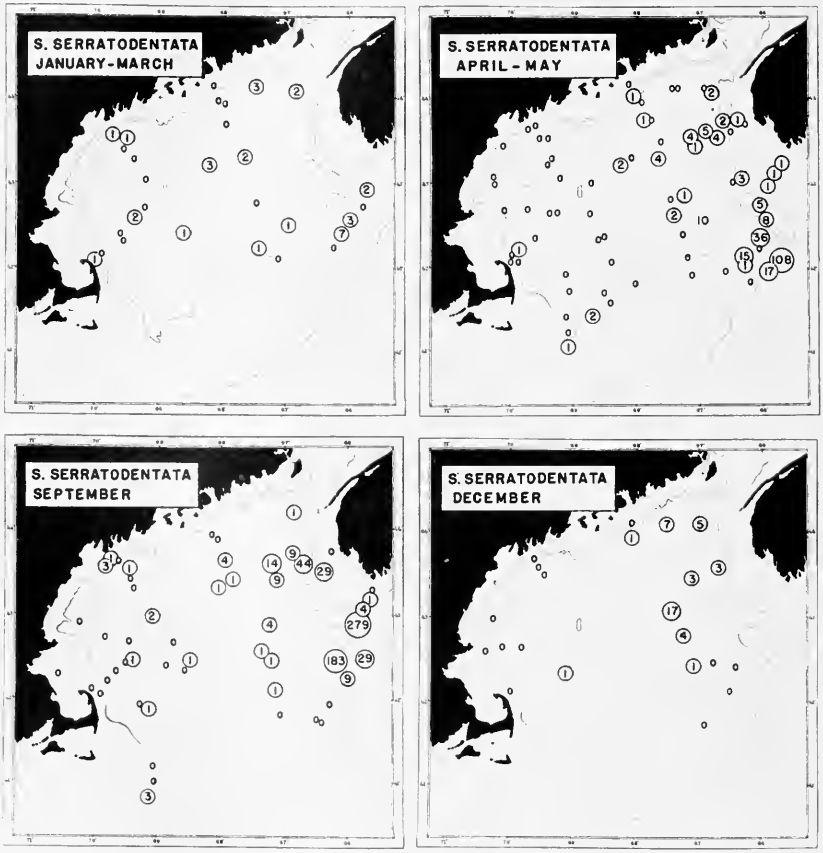

FIG. 9. Locations at which *Sagitta serratodentata* has been taken in the Gulf of Maine at different times of year. Figures in circles represent numbers taken per haul. Small circles represent positions of hauls in which none were taken.

the Gulf. Only in isolated cases were small numbers taken within the 100-meter contour or from the deep basin south and west of Cashes Ledge.

The seasonal picture during 1933–34 is given by Fig. 9, showing the combined catch in cruises grouped according to the time of year. Between January and March the population was at its low ebb, the distribu-

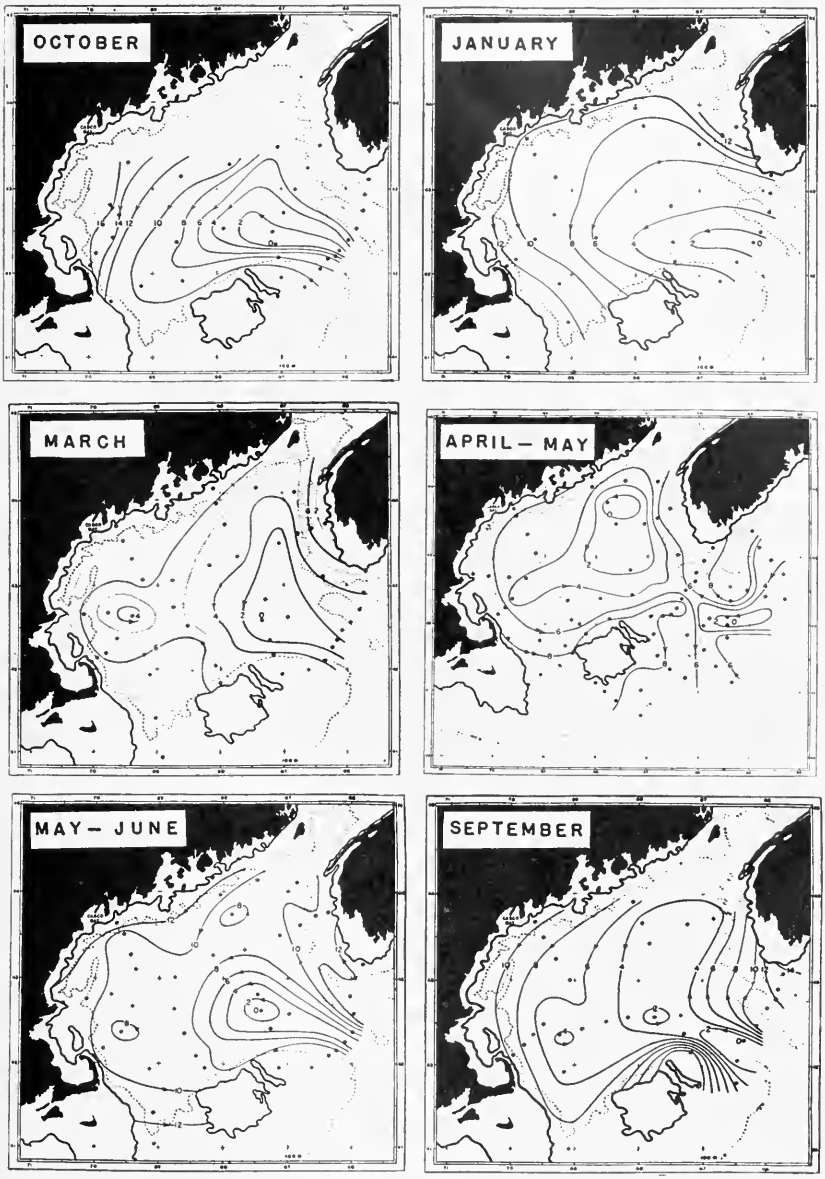

FIG. 10. Dynamic contours showing the theoretical circulation in the Gulf of Maine at a depth of 40 meters from October, 1933 to September, 1934.

tion was almost uniform throughout the Gulf. Such concentration as existed occurred in the northeastern quarter. By April and May the species had disappeared from the western side of the Gulf almost completely, but a new invasion was apparent from the southeast. By September the numbers had increased in this area of concentration and smaller numbers had extended westward as far as Portland while scattered individuals had again reached the remoter parts of the Gulf. By December the inflow of water carrying *S. serratodentata* appeared to have ceased and the remnants of the earlier invasion remain in small numbers still concentrated in the eastern region.

The course taken by *S. serratodentata* in its invasion of the Gulf is that expected of superficial water, entering from the outer Scotian Banks, in its circuit of the Gulf. It follows closely the drift of the population of *Limacina retroversa* which entered the Gulf in December, 1933 and circumnavigated the entire basin (Redfield, 1939). Unlike *Limacina,* this sagitta population did not survive to reach the western basin in significant numbers, and it was rarely found in the southwestern quarter, where lies the most ancient water in the Gulf. It also differed from *Limacina* in invading the Gulf continuously from late April through September.

Bigelow pointed out that neither temperature nor salinity explains the disappearance of *S. serratodentata* in the fall. The water is considerably warmer in November than it is at the time when invasion commences in the spring. Salinity is not very different then from that in late summer. He suggests that increases in numbers in the summer mirror the accumulation of a population which finds conditions favorable there. With the cessation of the invasion in autumn, the visitors of the summer die off from natural causes, leaving no progeny and so the species disappears until new immigrants enter in the spring. This explanation is clearly in accord with the picture we have obtained of the deep-water chaetognaths, save for the periodic character of the invasion.

Bigelow (1926) proposed that the periodic invasion of *S. serratodentata* may be connected with the seasonal reproductive cycle. The lack of any seasonal fluctuations in the numbers taken on the coastal belt west of Cape Cod, subsequently remarked by Bigelow and Sears (1939), leads us to suspect that the periodicity in the Gulf of Maine is due to a periodic change in the circulation of water, rather than to the seasonal character of the reproductive cycle.

The center of reproduction of *S. serratodentata* must lie in the warmer waters east of the continental slope, from which they penetrate the slope water and ultimately reach the Gulf in the indraft over the Eastern Channel. The dynamic contour charts (Fig. 10), showing the

theoretical pattern of circulation in the superficial layers, show that in April–May, 1934 an extensive area of dead water appears to occupy the banks south of Nova Scotia, and that the only apparent movement into the Gulf is from the continental margin off the Eastern Channel. In late May and through October this offshore indraft strengthened, thus supplying the required medium for the invasion of *S. serratodentata* into the Gulf. During the winter months the circulation of sub-surface water into the Gulf alters the contours indicating a much more intense flow across the Scotian Banks. Bigelow (1927) has shown that this change occurs with regularity and results in a regular invasion of water of low temperature and salinity. Such a flow will supply the Gulf with water derived from the inner and more eastern regions of the Scotian Banks where, according to Huntsman (1919), *S. serratodentata* is absent even in summer. During the winter months the invasion of this species into the Gulf will be checked and the population will decline until the inflow of this barren water across the Banks is checked, as it was in April–May, and the offshore indrift becomes predominant.

Sagitta elegans

Sagitta elegans Verrill is the most interesting member of the group because it is the only one maintaining a center of reproduction within the area. It is the characteristic sagitta of the north Atlantic coast, being of general occurrence in the shoaler water. Huntsman found it generally distributed in Canadian waters as far east as the Grand Banks, with particular abundance over the deeper parts of the coastal banks. Offshore it occurs only sparsely beyond the 100-meter contour. Georges Bank is the most southern important center of reproduction, though it ranges south as far as Chesapeake Bay in some seasons (Bigelow and Sears, 1939). In the Gulf of Maine Bigelow found *S. elegans* to occur most plentifully on Georges Bank, in the North Channel and on the adjoining banks south of Cape Sable, in Massachusetts Bay and in smaller numbers along the coast of Maine and New Hampshire within the 100-meter contour. Over the deep basin it is at all times much scarcer. This species becomes very scarce in most parts of the Gulf in early spring. Particularly, in Massachusetts Bay, where dense populations occur in the late summer, it almost entirely disappears in late winter.

Our collections confirm in detail the general features of the distribution of *S. elegans* in the Gulf of Maine described by Bigelow. By combining our data with his records for vertical hauls, and by grouping the two sets of observations by periods in which the findings are essentially

concordant, we are able to give in Fig. 11 a more detailed picture of the seasonal distribution than appeared from his analysis.

Georges Bank was the principal area of abundance of *S. elegans,* both in extent and density of population. Catches of hundreds or thou-

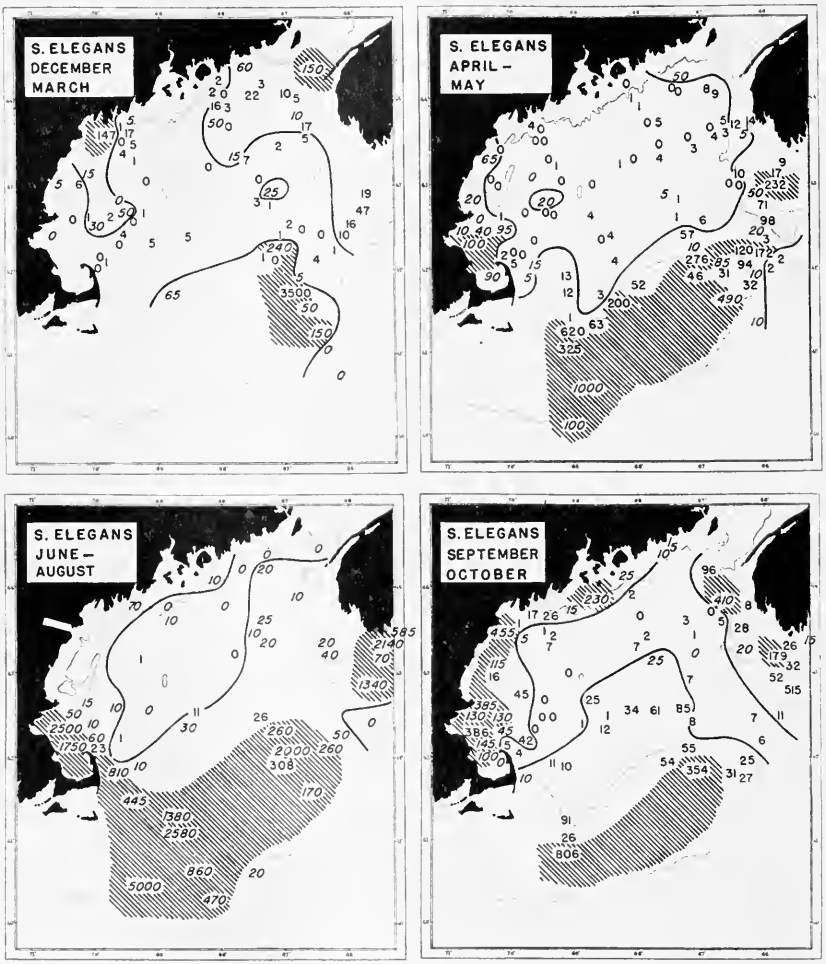

FIG. 11. Locations at which *Sagitta elegans* has been taken in the Gulf of Maine at different times of year. Upright figures represent numbers taken per haul by "Atlantis" in 1933–34. Italic figures represent numbers taken by Bigelow in vertical hauls. Contours enclose areas in which not more than ten specimens per haul were taken.

sands were taken there at all times of year. The area was not adequately explored during the winter months but on the eastern end, at

least, the catches were as abundant then as at any other time. One catch of 3,500 was made in December. The greatest abundance was observed in July.

The Scotian banks south of Cape Sable and the waters over the North Channel was the second area of abundance. In midsummer, catches were obtained there equalling those on Georges Bank. The numbers were less permanent, however, declining in late summer and becoming scanty during the winter period. Recovery was not indicated before May. This area appears to be an extension of the center of abundance which Huntsman (1919) observed on the western Nova Scotia banks in July.

Massachusetts Bay and the coastal waters extending eastward to the Bay of Fundy form an area of intermediate abundance, marked by great seasonal fluctuation. In winter *S. elegans* was rare in these waters with only scattered catches yielding important numbers. In May the population became augmented in Massachusetts and Cape Cod Bays. In midsummer populations rivalling those on the offshore banks developed here and increased numbers were found all along the coast to the eastward by early autumn. These numbers did not maintain themselves, however, with the coming of winter.

The waters over the deep basin of the Gulf and over the Eastern Channel were always scantily populated. There was not much fluctuation in numbers with season in the northern and western parts of the basin. In the southeastern half, particularly in the region north of Georges Bank, there was a distinct increase in the numbers taken, beginning in July and developing clearly in September.

We propose the following considerations in explanation of the observed distribution of *S. elegans* in these different regions. Georges Bank is the seat of a great anticyclonic eddy (Huntsman, 1924; Bigelow, 1927). The dynamic contours in Fig. 6 indicate the magnitude of this eddy. Because of its size and attendant permanence, the population of sagitta remains on the Bank in sufficient numbers to maintain a dense breeding stock. Fluctuations in numbers are only such as are to be expected in an animal reproducing periodically. The decline in numbers at the close of the reproductive season is to be attributed to the natural death of the parental generation and the loss of small numbers to adjoining bodies of water. This situation represents the most favorable condition for maintaining an endemic population. Since, as Russell (1932) has shown, *S. elegans* produces several broods per year, since these are not exactly synchronized, and there is only a short period of

reproductive inactivity in the early winter, fluctuations in numbers are too small to be discerned readily.[6]

The water south of Cape Sable is not recognized as forming a definite eddy, but it is spoken of as a "dead area" subject to strong tidal influence, but for considerable periods relatively free of residual drift. The dynamic contours presented in Fig. 10 show that this condition persisted from April through September in 1934. The freedom from strong currents enables the progeny of this population to accumulate in great numbers during the breeding season. At the end of the period of multiplication the population becomes rapidly scattered, as evidenced by the increased numbers over the coastal banks and deeper waters in the northeastern quarter of the Gulf in the fall and winter.

The alteration of the coastal currents during the winter, discussed in connection with *S. serratodentata,* contributes to the disintegration of this center of abundance. Adjoining extensive breeding areas to the eastward, however, this region remains populated throughout the year. Although the physical and biological conditions south of Cape Sable may be as favorable as on Georges Bank, and the areas to the eastward assure a constant supply of fresh immigrants, the hydrographic set-up does not have sufficient stability to maintain a rich population during the winter.

The coastal waters off the northern shores of the Gulf are the seat of a strong flow to the westward. A non-tidal drift of 7 miles per day is recorded at Portland lightship. Though conditions may not be unsuitable for reproduction in this area, the yield is swept on to the west and south. As a result, only in the relatively immobile waters of Massachusetts Bay is there an opportunity for large numbers to be produced by breeding early in the summer, while the stock grows more slowly in the region to the eastward. With the termination of multiplication the population of the entire coastal zone is rapidly reduced as the waters are swept on, and the population is incorporated into that of Georges Bank and the deeper basin to the north of it.

It is hard to believe that the deeper waters of the Gulf are scantily

[6] *S. elegans* appears to breed in the Gulf of Maine in the late spring and summer. Individuals less than 10 mm. in length may be taken from May to September in Massachusetts Bay and on Georges Bank. Huntsman and Reid (1921) consider the spawning season in the Bay of Fundy to extend from April to September, though the eggs do not develop properly until late summer and the authors doubt if conditions for reproduction are sufficiently favorable in that locality to enable the species to perpetuate itself except by immigration. In the Woods Hole region Fish (1925) found ripe eggs as early as March and April. Russell (1932) has detected four or possibly five successive broods of *S. elegans* in the English Channel, the first appearing as ripe adults in January, the last in October.

populated either because food is lacking or because its physical conditions are unfavorable. Plankton in general is rich in the deep basin, and though perhaps not as plentiful as on the banks, it would surely support more than one-thousandth as many sagitta. The differences in temperature and salinity in the surface waters are trivial. We suggest that the water over the basin is free from *S. elegans* for the same reason that it is populated with *S. serratodentata*. The reciprocal character of the distribution of the two forms is evident both from our own and Huntsman's observations. The superficial water lying over the deep basin of the Gulf is renewed from external sources, in which *S. serratodentata* occurs, but *S. elegans* is lacking. The flow of this water is sufficiently rapid at all times so that *S. elegans* becomes incorporated into it only in small numbers, particularly in the region of inflow and along the northern half of the Gulf. By the time the southern side of the Gulf is reached in the course of the flow about the great eddy which fills the Gulf, *S. serratodentata* has died off, but *S. elegans* has had time to penetrate in larger numbers into the waters overlying the deep basins. This penetration can occur, however, only at those times when a rich population has grown up in the coastal waters, that is, after midsummer, and when this supply is exhausted in early winter the offshore waters become depopulated, both from the death of their inhabitants and their transport out to sea.

Discussion

We have attempted to analyze the distribution and abundance of chaetognaths in the Gulf of Maine on the basis of certain biological characteristics of the species and the movement of the water in which they live. We believe the character of their distribution within the area studied can be accounted for in the main in these terms without recourse to considerations of the suitability of the physical characteristics of the water for survival of the adults or of nutritive conditions.

The biological characteristics taken as given are the depth of water which the species frequents and the character of the water in which it succeeds in breeding. The former is doubtless controlled by choice, tropisms, or avoidance reactions on the part of the individual. Such reactions are left out of account as necessary factors in determining horizontal distribution, it being sufficient to consider that this is determined by the flow and mixing of the water masses in which the animals live. Active swimmers such as the chaetognaths might be capable of definite migrations such as many fish perform. There is no evidence that such directed movements influence the distribution of these forms, though Meek (1928) has claimed that cross-current migrations do influ-

ence the character of the sagitta population along the Northumbrian coast. In the absence of directed migrations, their swimming may be expected merely to accentuate the scattering of the population in horizontal directions at rates greater than the concurrent mixing processes in the water itself.

The situations in which a species can breed are, of course, controlled in some way by the physical characteristics of the water, particularly its temperature. Many species are able to survive as adults in regions in which reproduction is not possible. Five of the six chaetognaths considered fall into this category in the Gulf of Maine. Success in breeding is itself a relative matter and in "marginal" regions insufficient eggs may develop to maintain the local stock. *S. elegans* in the Bay of Fundy appears to be a case in point. There Huntsman and Reid (1921) find that the conditions are unfavorable for the development of larvae and conclude that the very considerable fluctuations in their numbers are caused by the adults being carried into the Bay in varying quantities. We may classify each species as it occurs in a given locality in an order of fecundity which decreases in its favorable effect on the maintenance of the population thus: (1) successful breeder; (2) unsuccessful breeder; (3) non-breeding immigrant.

These categories are merely relative. Success in breeding will affect the abundance of the population both in proportion to the number of eggs spawned and the fraction of these which survive.

Fluctuations in the stock present at any time will depend in part on the purely biological facts relating to the breeding habits. Animals having an annual period of reproduction will show a marked fluctuation with maximal numbers immediately following the period of breeding. The fluctuations will be most marked if the adult does not long survive its first breeding, less pronounced if the adult survives through several breeding seasons. Species which maintain their abundance by the production of many eggs with small expectancy of survival will fluctuate more than those less fecund species with low mortality. The degree to which the reproduction of different species is synchronized with the season will also modify the seasonal incidence. The life histories of the sagittae are not sufficiently well known to permit of classification in these regards. Fluctuations appear to be relatively small in *S. elegans* on Georges Bank, the most stable region of its occurrence. The deep-water species are free from marked fluctuation as is *S. serratodentata* according to Bigelow and Sears (1939) in its reproductive area south of Cape Cod. The marked fluctuations in the latter and the periodic occurrence of *S. enflata* appear to depend on environmental causes, rather than on the biology of the reproductive cycle.

A final biological factor determining both abundance and distribution is longevity. Within a region of production the relation of mortality to reproductive rate is obvious and has been mentioned above in considering fluctuations of biological origin. In the case of immigrant plankton, which are carried beyond the areas of successful breeding, the longevity of the individuals will determine the distance which may be travelled and the relative numbers to be found in regions increasingly remote from the centers of production. The deep-water chaetognaths afford examples of this sort as do *S. serratodentata* and *S. lyra*.

In nature these biological factors are at play in a scene determined by the given hydrography of various regions. The permanence of a maximal population demands that the breeding stock shall not be scattered by the flow of water, or that such losses shall be balanced by recruitment from other rich areas. The most favorable conditions for endemic existence will be found in large areas where suitable conditions are found and in which the water remains more or less permanently. Such an area is provided by the great eddy which appears to exist on Georges Bank and here *S. elegans* maintains a dense and stable population in spite of the fact that there is little opportunity of recruitment from other regions. Russell (1936*b*) considers the source of the water which occasionally carries *S. elegans* into the English Channel to be an area of cyclonic circulation lying south of Ireland.

When opportunities for recruitment by immigration from adjoining areas are good, rich populations may be developed in regions which do not develop permanent eddies in spite of a relatively rapid dissipation, as in the case of the banks south of Cape Sable. In such areas the seasonal fluctuations become greater and increasingly so in small areas such as Massachusetts Bay in which little time is required to wash away the dense populations, as soon as the period of multiplication is passed. In such regions the population is pseudo-endemic inasmuch as it is dependent on immigration to a high degree.

Bodies of water originating in regions where a species cannot maintain a production center may acquire a population by immigration from such centers in the course of its drift. The scanty populations of *S. elegans* which are found in the waters over the central basin of the Gulf of Maine appear to be of this type. Such populations become richer the longer the water is in proximity to the source area of production. Whether the species can reproduce in the area or not, it cannot achieve significant numbers because the water moves on too rapidly to permit an accumulation. Such populations may show seasonal fluctuations reflecting the reproduction periods of the areas from which they are derived, as in the southern half of the basin.

The origin of water bodies having distinctive physical characteristics results primarily from the mixing of other characteristic waters. The formation of slope water through the admixture of tropical, boreal and coastal waters is a case in point. In its synthesis the inhabitants of the contributing regions become incorporated into it and are carried with it to regions remote from their centers of reproduction. Such immigrants, if unable to reproduce themselves, can travel only so far as their life span will permit. They may be regarded as terminal immigrants in contrast to the forms such as *S. elegans* in Massachusetts Bay which form pseudo-endemic populations in regions continuously swept out by the passing waters. *E. hamata, S. maxima* and *S. lyra* are examples of this category.

Immigrants, both terminal and pseudoendemic, may show fluctuations both as the result of the reproductive cycle and for hydrographic causes. While the fluctuation of the pseudo-endemic population of *S. elegans* in Massachusetts Bay is probably due to seasonal reproduction in an area of relatively constant dissipation by currents, a changing current pattern may contribute largely to the fluctuation of the population of this species off Cape Sable. *Limacina retroversa* fluctuates greatly in numbers in the Gulf because it enters intermittently presumably because of fluctuations in the sources of the water entering the Gulf. Though it may be classed as an unsuccessful breeder in the Gulf, the important reason for its failure to develop a truly endemic population is the rapid flow of water across the surface of the basin which carries it away following its intermittent periods of invasion. *Limacina retroversa* is thus an unsuccessful pseudo-endemic form fluctuating for hydrographic reasons.

S. serratodentata, essentially a terminal immigrant in the Gulf of Maine, fluctuates in its occurrence for reasons which appear to be largely hydrographic. Being unable to survive long after the cessation of the inflow which introduces it into the Gulf, its fluctuations so far as this area is concerned are more marked than any others. *S. enflata,* occurring as a rare straggler along the borders of the Gulf, is the extreme case of terminal immigration. Its occurrence in late summer, along with many other tropical forms, suggests a definite movement of tropical water onto our shores at that time. The water in which these forms occur does not differ from normal coastal water in salinity. Their presence is due to the admixture of exceedingly small amounts of tropical water into that of the coastal banks. Their seasonal occurrence is to be related to the annual temperature cycle of the coastal waters. Unable to survive the chilling of winter, these rare stragglers are unable to travel far from their production areas beyond the slope water until the latter part of summer.

Among the chaetognaths, only one—*S. elegans*—has established a truly endemic existence in the Gulf of Maine, and this on the coastal and offshore banks. It alone achieves important numbers for this reason, and it alone is sufficiently numerous so that nutritive conditions may limit its numbers. The other species are immigrants from other regions and occur in numbers depending on the remoteness of their areas of production and no doubt on their abundance in such regions.

We may inquire why the basin of the Gulf has acquired no successful endemic population of chaetognaths. So far as the surface waters are concerned, the answer seems to be that these circulate so rapidly that time is not permitted for the accumulation of even a considerable pseudo-endemic population. The evidence is found in the history of *Limacina retroversa* and of *S. elegans* in the southern half of the basin. In the deeper water this difficulty can scarcely obtain. These waters derived from the slope water undergo rapid modification within the Gulf by admixture with surface water and acquire lower temperatures and salinities than those characterizing the slope water at comparable depths, and lower salinities than slope water of the same temperature. The process of creating a characteristic water mass, such as the slope water is, is continuing actively in the basin of the Gulf. Perhaps for this reason no chaetognath has yet appeared capable of reproducing successfully in this unique and limited region. However this may be, it is clear that the basin of the Gulf does support a rich endemic population of Crustacea. How this is possible should be the subject of future study.

Summary

1. Data are presented concerning the distribution and numerical abundance of five species of chaetognaths taken in the Gulf of Maine during the year 1933–34 and the hydrographic features controlling their abundance is discussed.

2. It is concluded that *Eukrohnia hamata, Sagitta maxima* and *S. lyra,* which are carried into the Gulf by deep currents and do not breed there, occur in numbers which depend not only on their relative abundance in various offshore waters which mingle in the Gulf, but on their longevity after entering the Gulf.

3. *Sagitta serratodentata* is a terminal immigrant from the superficial waters of the Atlantic which fluctuates in its abundance as the result of periodic changes in the circulation of water entering the Gulf from the east.

4. *Sagitta elegans* is the only chaetognath truly endemic to the region. The permanence of its occurrence appears to depend on the presence of

a relatively stable eddy on Georges Bank. Its occurrence in other regions varies with the season to a degree which may be explained by local conditions of circulation.

5. *Sagitta enflata* is recorded for the first time from the margins of the Gulf of Maine.

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STUDIES ON THE PROTEINS OF SMOOTH MUSCLE

II. The Myosins of the Octopus, Snail, Sea Cucumber and Sea Anemone

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Despite great advances in our knowledge of the composition of muscle, and of the chemical reactions which accompany contraction, it cannot yet be said that we have any real understanding of the process of muscular contraction. One of the obstacles in the way of such an understanding is our lack of knowledge regarding the position of the muscle proteins, and their rôle in contraction. From the standpoint of structure, myosin appears to be the most important protein in muscle. The available evidence indicates that myosin accounts for from 40 to 50 per cent of the total proteins of muscle (1) (2) (3) (4). Both the optical properties and the X-ray diffraction patterns of muscle fibres and myosin fibres show a striking correspondence (5) (6) (7) (8) (9). It would therefore appear reasonable to suppose that the muscle fibril is essentially a fibre of oriented myosin molecules, and that contraction is associated with a contraction of this fibre. There are, however, a number of unsolved problems, such as the significance of the anistropic and isotropic bands (striations) in striated muscle and the existence or non-existence of certain membranes across the fibre axis. Some aspects of these problems have been discussed by Weber (3) (4) and by Bernal (10).

Because of its apparently greater structural simplicity, smooth muscle might seem to be a better system for investigation. Under any circumstances, any general theory of the place of myosin or other proteins in muscular contraction must consider the properties of both smooth and striated muscle. In particular, it seems desirable to extend our information regarding the properties and distribution of myosin. Previous studies have been limited largely to the striated muscle of the vertebrates, although von Muralt and Edsall (11) have reported the isolation of myosin from the smooth muscle of the marine snail, *Busycon*. More recently myosin has been prepared from lobster muscle (12). Bailey also studied the composition of the myosins of skeletal muscle of several

[1] These studies were made at Kerckhoff Marine Laboratory of the California Institute of Technology, Corona Del Mar, California.

species of vertebrates. In addition to the study of composition, it would be desirable to investigate the variation of the physical properties of myosin in different animals and in different types of muscle.

Two very characteristic properties, which serve to distinguish myosin from other muscle proteins, are its solubility and double refraction of flow. The solubility properties of myosin make the preparation a relatively simple process; while the double refraction of flow, which is indicative of the rod, or thread-like shape of the myosin molecule, may be used to characterize the preparations obtained. With the construction of a portable apparatus for the study of double refraction of flow, a brief, exploratory excursion into marine biology seemed attractive. The results obtained, though limited, indicate certain interesting possibilities and problems.

Methods

The preparation of myosin is based upon its characteristic solubility properties (1) (2) (3) (13) (14) (15). Myosin is insoluble in salt solutions and water at pH 5 to 6, but soluble in moderately concentrated salt solutions on the alkaline side of the isoelectric point. It is, however, relatively insoluble in dilute salt solutions at pH 6 to 7. In the method used, the tissue is thoroughly minced and extracted with 0.5 to 1 M KCl which contains 0.02 M $NaHCO_3$ or phosphate buffer with a pH of 7 or greater. The extract is filtered through cheesecloth and then through paper pulp. The filtrate is then adjusted to about pH 6.5 and diluted with 5 to 10 volumes of water. This should cause the myosin to precipitate in a flocculent form which will settle out slowly. After this preliminary concentration of the precipitate by settling, it can be further concentrated by centrifugation. The precipitate is washed with water, and is finally dissolved by adding sufficient saturated KCl solution to give a solution of 0.5 M to 1 M. It is desirable to carry out all the procedures in the cold, but in the present experiments only the extraction and the preliminary concentration of the precipitated myosin by settling could be done in the cold. Recent studies (15) (16) have shown that the behavior of myosin preparations, particularly with respect to double refraction of flow, may be markedly influenced by a variety of factors. Most salts have a considerable effect, and even KCl in too high concentrations may result in reduced double refraction of flow. For that reason, the data presented cannot be assumed to represent values for completely unaltered myosin. A more detailed study of each myosin will be needed to determine the particularly important factors in each case.

The apparatus used for the study of double refraction of flow is, in

principle, the same as that previously used in this laboratory (11) (17) (25).[2] Solutions which show double refraction of flow,—that is, become optically anisotropic when subjected to shearing forces,—may be best studied by placing them between two concentric cylinders, one of which rotates, while the other is held fixed. The shearing forces resulting from the velocity gradient produced in the liquid in this way will be uniform along any line through the liquid, parallel to the axis of the cylinders. The optical properties will therefore be uniform along such a line, and may be conveniently observed with a suitable optical system. The principles of these measurements are discussed in the papers referred to above (11) (17) (25), as well as in a number of other papers (19) (20) (24).

Two variables are studied as a function of the velocity gradient, the extinction angle, and the amount of double refraction. The extinction angle is the angle between the optical axis in the bi-refringent solution and the tangent to the circumference of the concentric cylinders at the radius on which the point of observation lies. According to the theory of double refraction of flow (20) (21) (22), the direction of the optical axis will generally correspond to the principal direction of orientation of the molecules, so the extinction angle will give the angle between the principal direction of orientation of the long axes of the molecules and the tangent to the circumference of the cylinders. When the velocity gradient approaches zero, the orientation becomes more nearly random, and the extinction angle approaches 45°. At higher velocity gradients the molecules become more highly oriented, and tend to lie with their long axes parallel to the tangent to the circumference. Thus, at high velocity gradients, the extinction angle will approach 0° as a limiting value. The ease with which the molecules may be oriented depends upon their size and shape, and an approximate relation has been obtained for the extinction angle, χ, as a function of the ratio of the velocity gradient, β, to the rotatory diffusion constant, Θ (20) (21). With reference to the results to be presented, it may simply be said that the longer the molecule, the smaller the extinction angle will be at a given velocity gradient. Since the rotatory diffusion constant is roughly inversely proportional to the cube of the length of the molecule, the extinction angle will be quite sensitive to the length of the molecule.

The amount of double refraction is given by the difference between the refractive indices of the solution for the extraordinary and for the ordinary ray, $n_e - n_o$. The value of $n_e - n_o$ is zero for zero velocity gradient, and increases toward some upper limit as the velocity gradient

[2] A complete description of the new apparatus will be given in a future publication.

is increased. The upper limit corresponds to the value of $n_e - n_o$ for complete orientation of all the molecules. Practically, it is not possible to reach this upper limit, nor can it be readily calculated; but it will depend upon the specific optical properties of the molecules,—upon their refractive index and that solvent, and upon any double refraction inherent in the molecule itself. The amount of double refraction will also depend upon the concentration of the solution, and in dilute solution will be proportional to the concentration.

It will be seen that the extinction angle is a function, at least in dilute solution, of the velocity gradient and the rotatory diffusion constant alone. It is, therefore, the most useful value for estimating the size and shape of the molecule under consideration. The amount of double refraction is a function of the optical properties as well, and cannot be used alone to obtain information regarding either size and shape, or the optical properties of the molecule. When, however, different solutions are compared at equal values of the extinction angle (that is, at equal degrees of orientation), a comparison of the amounts of double refraction does give some indication of the inherent optical properties of the molecules. Measurements of $n_e - n_o$ are also useful for measurements of the rate of loss of double refraction of flow under various conditions.

The nitrogen determinations were made by the micro-Kjeldahl method. pH determinations were, for the most part, very roughly made, using indicators. In several cases, however, these values were checked by measurements with the glass electrode.

Materials

The Sting Ray (Urobatis halleri)

The tail and wing muscles [3] of one ray were ground and extracted in the cold with 1 M KCl + 0.02 M $NaHCO_3$. After precipitation of the myosin it was dissolved by adding KCl to make the solution 1 M. The solution was very turbid and was not readily clarified, so only a limited number of experiments were made with it.

The Octopus (Polypus bimaculatus)

The first preparation was made by extracting the ground tentacles with 1 M KCl + 0.02 M $NaHCO_3$. Considerable difficulty was experienced with turbidity in the filtrates, and when this myosin was finally redissolved in 1 M KCl it showed no double refraction of flow. A second preparation was then made, this time using 0.5 M KCl + 0.02 N $NaHCO_3$ for the extraction. This gave a solution of myosin which,

[3] These muscles are striated.

though turbid, showed strong double refraction of flow. Precipitation upon dilution occurred at a pH as high as 7, although the point of maximum flocculation upon the addition of acid was around pH 4.5 to 5.5.

The Snail (Polynices lewisii)

The foot muscle from two snails was ground and extracted with 0.5 M KCl + 0.02 M $NaHCO_3$. In adjusting the pH for precipitation there was a very marked tendency for precipitate to form at the addition of each drop of acid, and a precipitate could be obtained upon dilution at a pH as high as 8. The solution of myosin finally obtained was quite turbid, but showed marked double refraction of flow.

The Sea Anemone (Cribrina xantho-grammica)

The whole animal was ground and extracted with 0.5 M KCl + 0.02 M $NaHCO_3$. The filtrate showed no double refraction of flow, and acidification with HOAc gave only a small amount of precipitate. The precipitate would not redissolve in KCl, and seemed to be due to the aggregation of suspended material.

The Sea Cucumber (Stichopus californicus)

The cutis and muscularis were treated separately. Each was extracted with 0.5 M KCl + 0.02 M PO_4 at pH 7.5. Neither extract showed double refraction of flow, any precipitate on dilution at pH 6.5, or more than a small amount of precipitation at pH 5. A second extract was made, this time using phosphate buffer with a pH of 8.5, but these extracts behaved like the first pair.

Results

The measurements of the extinction position in the solution are shown in Fig. 1. The extinction angle is given as a function of the velocity gradient within the solution. All of these measurements were made in concentrations at which the position of the optical axis is affected somewhat by concentration, so solutions of about the same concentration have been chosen for comparison. The measurements on the octopus and snail myosins were made at about 25° C., and are not strictly comparable with the values for rabbit myosin,[4] which were made at 3° (17). The values for Ray myosin,[5] which are not shown, would indicate that it is similar to octopus myosin.

[4] The muscles from which the myosins of the sting ray and rabbit were prepared are striated.

[5] The isolation of myosin from mammalian intestinal muscle has been reported (27). Certain atypical properties make it seem desirable to withhold final judgment on that case pending further investigation.

Figure 2 gives the values of the amount of double refraction of flow as a function of the velocity gradient. Because of the approximate proportionality between the amount of double refraction at a given velocity gradient and the concentration, the values of $n_e - n_o$ have been divided by the concentration of the protein in grams per cc. of solution, using a nitrogen factor of 6.45. The snail myosin gives values which are higher than those for the octopus myosin at equal velocity gradients. This is due, however, to the greater degree of orientation of the snail myosin. If the values of the double refraction are compared at equal values of the extinction angle, the double refraction of the octopus myosin would

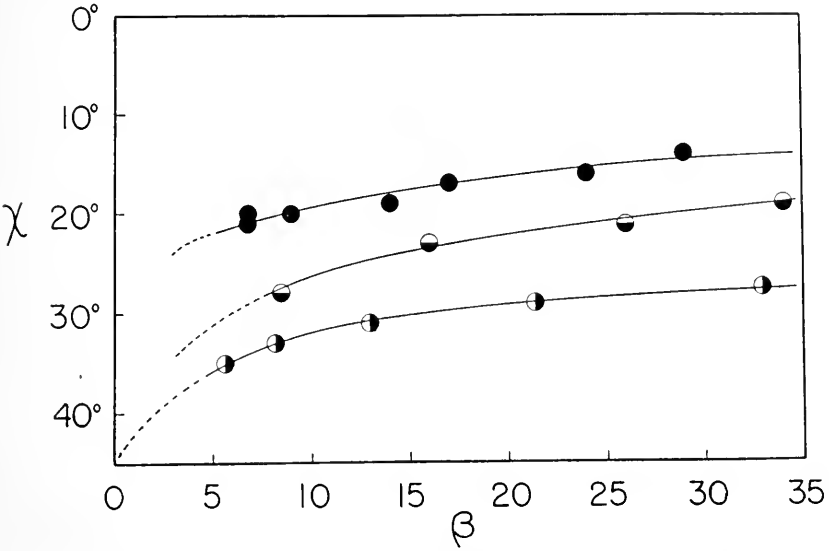

FIG. 1. The extinction angle, χ, as a function of the velocity gradient, β. ●—snail myosin at 25°, nitrogen = 5.3×10^{-4} gram/cc.; ◑—octopus myosin at 25°, nitrogen = 5.9×10^{-4} gram/cc., 25°; ◒—rabbit myosin at 3°, 4.5×10^{-4} gram/cc., from the measurements of von Muralt and Edsall (17).

seem to be greater. Moreover, the value of the double refraction of the snail myosin is about equal to, or slightly greater than, that of the rabbit myosin at equal values of the extinction position. Here again the measurements on the rabbit myosin were made at 3° (11), while the measurements of this investigation were made at 25°. The effect of temperature upon the amount of double refraction is quite marked (11), and the change from 3° to 25° would reduce the double refraction by about 50 per cent. Cow myosin gives values of the angle of isocline comparable with rabbit myosin, but has about twice the amount of double refraction (11) (17).

The effect of alkali on these myosins is similar to the effects already reported for cow and rabbit myosin (11). Measurements of the amount of double refraction were made at constant velocity gradient, as a function of time. The double refraction of flow decreases rapidly at pH 10. A decrease is obtained with octopus myosin at pH 8, the loss being about 50 per cent in 45 minutes. At pH 9 a decrease to 50 per cent of the initial value takes place in about 25 minutes. Essentially the same result was obtained with snail myosin.

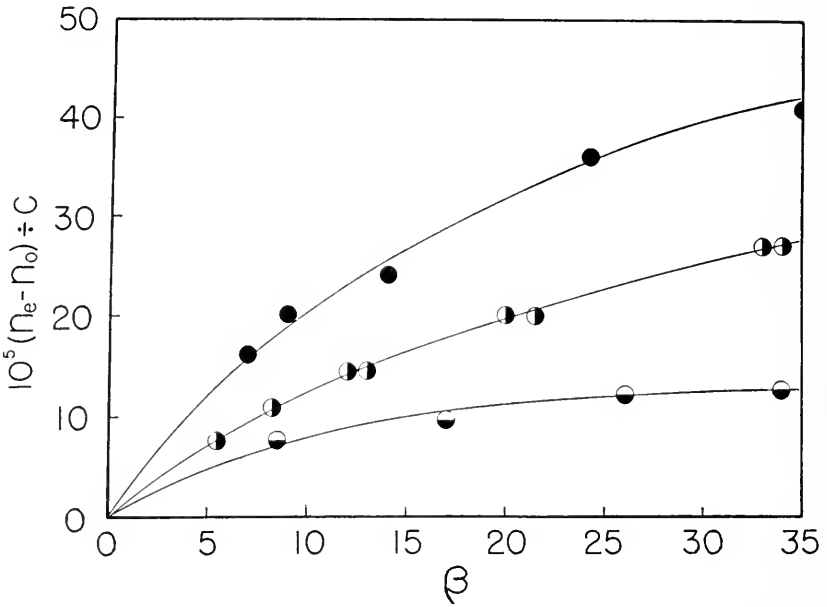

FIG. 2. The amount of double refraction $(n_e - n_o) \div C$, as a function of the velocity gradient, β. ●—snail myosin at 25°, nitrogen = 5.3×10^{-4} gram/cc.; ◑—octopus myosin at 25°, nitrogen = 5.9×10^{-4} gram/cc.; ◒—rabbit myosin at 3°, nitrogen = 4.5×10^{-4} gram/cc., from the measurements of von Muralt and Edsall (11).

The effect of urea upon the double refraction of flow was also studied. The results obtained are shown in Fig. 3, where the percentage of the original amount of double refraction is given as a function of the time. There is a general similarity between the different myosins, with secondary differences. It is perhaps of some interest that the ray myosin is no less sensitive to the effects of urea than are the other myosins, although the uremia of the elasmobranchs (about 2 per cent urea in marine elasmobranchs) is of the same order of magnitude as the concentrations of urea needed to produce denaturation (about 3–4 per cent).

Discussion

Perhaps the most notable feature of these results is that the differences between the mollusk myosins and the vertebrate myosins are no

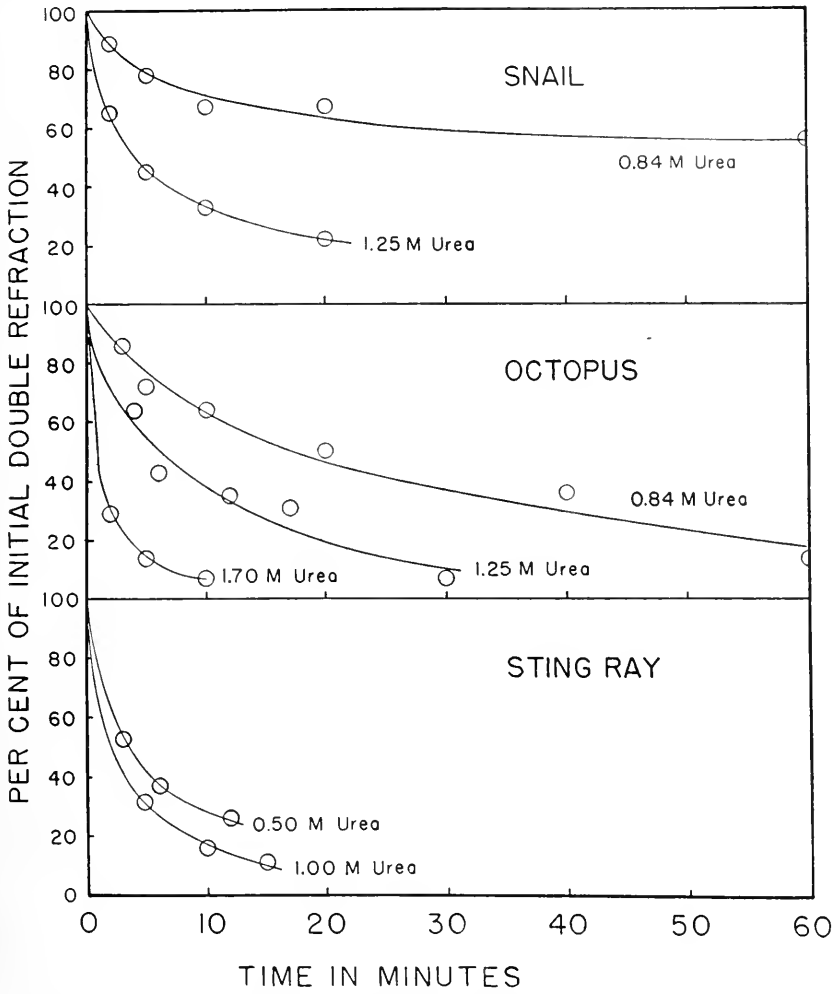

Fig. 3. The effect of urea upon the amount of double refraction of flow. Percentage of original double refraction of flow as a function of time after mixing with urea to give the final concentration of urea indicated.

greater than the differences between the myosins from the two species of mollusks, or between the different vertebrate myosins. From measurements of double refraction of flow, it has been estimated that the length

of the rabbit myosin molecule is between 5,000 and 10,000 A (25). Calculations made in the same way would place the length of the octopus myosin at 5,000 to 10,000 A, the snail myosin at 13,000 to 27,000 A, and the ray myosin at 5,000 to 10,000 A. This is in keeping with the general similarity in the amino acid composition of the various myosins. This similarity in composition, the characteristic solubility, and the common large size and great asymmetry indicated by double refraction of flow, all point to a common architectural plan. The fact that the same protein is found in both smooth and striated muscle would indicate that, at least in certain cases,[5] the two types of muscle differ with respect to the arrangement of the proteins within them rather than with respect to the nature of the proteins of which they are composed.

The failure to isolate such a protein from the sea cucumber and the anemone would be as interesting and significant if it were not possible that the negative result is due to the protein being less accessible to extraction—perhaps because disintegration of the cells is more difficult. In the case of the sea cucumber cutis, the X-ray results of Astbury and co-workers (28) make it seem likely that the doubly refracting fibres are composed of a protein of the collagen group. Further work is necessary to elucidate the position of the sea cucumber muscle, and to determine whether myosin will be found in other invertebrate muscles. It would seem to be particularly important to investigate the possibility of a correlation between the properties of muscle proteins and the physiological properties of muscle.

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NOTES ON THE PHARMACOLOGY OF THE HEARTS OF ARIOLIMAX COLUMBIANUS AND ASTACUS TROWBRIDGEI

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In the Mollusca the mediator acetylcholine has been found to be a cardiac inhibitor, while in the Arthropoda it is strongly stimulatory to cardiac action. Atropine has been found to abolish the action of acetylcholine in Arthropoda (Welsh, 1939*a*, 1939*b*; Hamilton, 1939) but not in Mollusca (Jullien, 1936; Prosser, 1940, etc.). In both groups, as well as in many other invertebrates and vertebrates, it has been found that eserine sensitizes muscle tissue to acetylcholine by rendering inactive the cholinesterase present in the tissue, and perhaps because there exists a synergism between acetylcholine and eserine (Freud and Uyldert, 1936). Nicotine is known to act on ganglionic cells, thus blocking nerve impulses. In the vertebrates in small doses it leads to temporary stimulation followed by depression, and in large doses depression is evident immediately.

Ariolimax columbianus

The investigation of molluscan hearts to determine whether or not a cholinergic system of heart control is present has occupied the interest of physiologists for several years. The mollusks are a diverse group; this fact is emphasized by consideration of a brief tabulation of findings regarding the cholinergic physiology of the hearts of various forms (Table I). It is interesting that Prosser (1940) finds that the sensitivity of the heart of the clam, *Venus*, to acetylcholine varies greatly from spring to fall. Included in the table are the experimental results for the slug *Ariolimax*.

In the slug the heart, kidney and lung make up the pallial complex which almost fills the mantle cavity. The heart, which lies on the dorsal surface of the kidney, consists of a very thin-walled auricle and a ventricle from which issues a large aorta; it is essentially a muscular tube constricted at one point.

The animal is fastened to wax with pins through the foot at 2 or 3 cm. intervals. A small incision is made under the posterior flap of the

mantle about 1 cm. to the left of the median line; the cut is carried forward to the anterior edge of the mantle. The skin on the right side of the incision is lifted and cut away as necessary, disclosing the delicate pericardium, under which can be seen the beating heart. In cutting the pericardium, great care must be taken not to damage the ventricle, which responds to harsh, direct contact or injury by going into systolic tetany.

The aorta is cut about 5 mm. from its origin, and the heart is freed from pericardial and renal tissue; the auricle is severed about 3 mm. from the auriculo-ventricular junction, and the ventricle is transferred immediately to the physiological solution. The aortal stub is secured to a small steel hook fixed in a bent glass rod, and a small glass hook suspended by a thread from the recording lever is passed through a hole made through the remaining auricular tissue. When tension is applied, the ventricle beats rhythmically, shortening along the vertical axis.

The isolated ventricle is completely bathed in a desired solution by raising a beaker containing that solution around the support of the fixed steel hook. By using this method it is possible to change the solution quickly and smoothly without disturbing the record.

The most favorable salt solution used was Cardot's (1921) *Helix* Ringer. Salts were dissolved in distilled water in the following concentrations: (grams per liter) NaCl 9.0; KCl 0.42; $CaCl_2$ 0.24. Possibly this Ringer did not have a perfect ionic balance nor complete isotonicity to the plasma of *Ariolimax*; the ventricle remained active for only a few hours after excision.

The temperature effects mentioned by Carlson (1906) were found to hold true in these experiments. At about 16° C. the normal heart rate consisted of about 35 ventricular systoles per minute; at 22° C. there were 57 beats per minute. Thus, for every degree rise in temperature, there was an increase of about 3.6 beats per minute. Recording was done in a cold-room with temperature of 14–18° C. The temperature remained constant during a given experiment; uniform rate of beat was thus insured.

Acetylcholine chloride Merck was kept on ice in stock solutions of .1 gram in 100 cc. of .01 N HCl. Aqueous stock solutions of 1:1000 eserine salicylate Merck, atropine sulphate Merck, and nicotine Eastman were also kept on ice, and with acetylcholine were freshly prepared every 2 or 3 days.

Acetylcholine

Acetylcholine has negative inotropic and negative chronotropic effects on the ventricle of *Ariolimax*. The first definite indication of an effect is seen at 1:10 million (Fig. 1*B*), and this concentration may be

TABLE I

The effect of drugs on molluscan hearts.

Animal	Acetylcholine Effect	Acetylcholine Effect after Eserine	Atropine Effect	Acetylcholine Effect after Atropine
Oyster (Jullien, 1936)	Threshold 1 : 100,000. Diastolic stoppage between 1 : 50,000 and 1 : 5,000.	***	1 : 1000 gives pos. ino- and neg. chronotropic effects.	No antagonism
Clam *Venus mercenaria* (Prosser, 1940)	Threshold 1 : 1 trillion in spring; 1 : 10 billion in fall.	Increased sensitivity to acetylcholine. Prolonged inhibition by acetylcholine.	1 : 10,000 toxic	No antagonism
Snail *Helix sp.* (Jullien, 1936)	Threshold 1 : 1 million. Diastolic stoppage at 1 : 50,000	***	1 : 20,000 gives pos. chrono- and small neg. inotropic effects.	No antagonism
Snail *Murex sp.* (Jullien, 1936)	Threshold 1 : 1 million. Diastolic stoppage at 1 : 50,000	***	1 : 2500 starts heart which has not started to beat.	No antagonism
Slug *Ariolimax columbianus*	Threshold 1 : 10 million. Diastolic stoppage at 1 : 50,000.	Increased sensitivity to acetylcholine	1 : 1000 toxic	No antagonism
Squid *Loligo pealii* (Bacq, 1934)	Threshold 1 : 1 billion. Diastolic stoppage at 1 : 10 million.	***	1 : 1 million gives negative inotropic and chronotropic effects.	No antagonism

considered the threshold. At progressively stronger concentrations the effects become more pronounced until at a concentration of 1 : 50,000 diastolic stoppage occurs (Fig. 1*C*). Normal beat is always resumed upon replacement of the acetylcholine solution by Ringer. After diastolic stoppage of a heart by acetylcholine, direct local electrical stimulation causes a strong contraction.

Eserine

A heart was bathed for 20 minutes in a 1 : 1 million solution of eserine, which has only a slight slowing effect; during this time the solution was frequently washed. In this eserinized heart the acetylcholine threshold was lowered to 1 : 100 million (Fig. 1*D*). Diastolic stoppage oc-

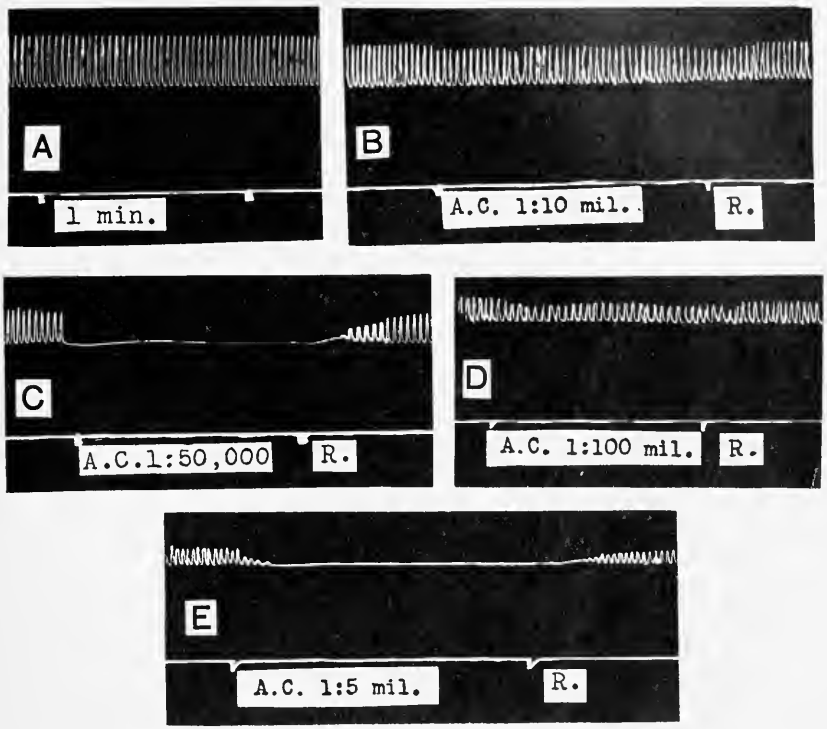

FIG. 1. *Ariolimax*. *A*, normal beat at 16° C. *B*, effect of acetylcholine 1 : 10,000,000. *C*, effect of acetylcholine 1 : 50,000. *D*, effect of acetylcholine 1 : 100,000,000 on ventricle treated for 20 minutes with eserine 1 : 1,000,000. *E*, effect of acetylcholine 1 : 5,000,000 on ventricle treated for 20 minutes with eserine 1 : 1,000,000.

curred with acetylcholine 1 : 5 million (Fig. 1*E*), that is, with a solution of acetylcholine 100 times more dilute than is necessary for stoppage of an un-eserinized heart.

Atropine

Atropine has no effect except in toxic concentration (1 : 1000). After administration of a non-toxic solution of atropine (1 : 10,000) or in

mixture with such a solution (Fig. 2*A*), acetylcholine 1 : 50,000 carries out its characteristic stoppage effect without alteration.

Nicotine

Positive inotropic and negative chronotropic effects were produced by this drug in low concentrations. The first noticeable effect was a slowing of the rhythm by nicotine 1 : 7000. With nicotine 1 :5000 a definite negative chronotropic effect could be observed, and to this effect

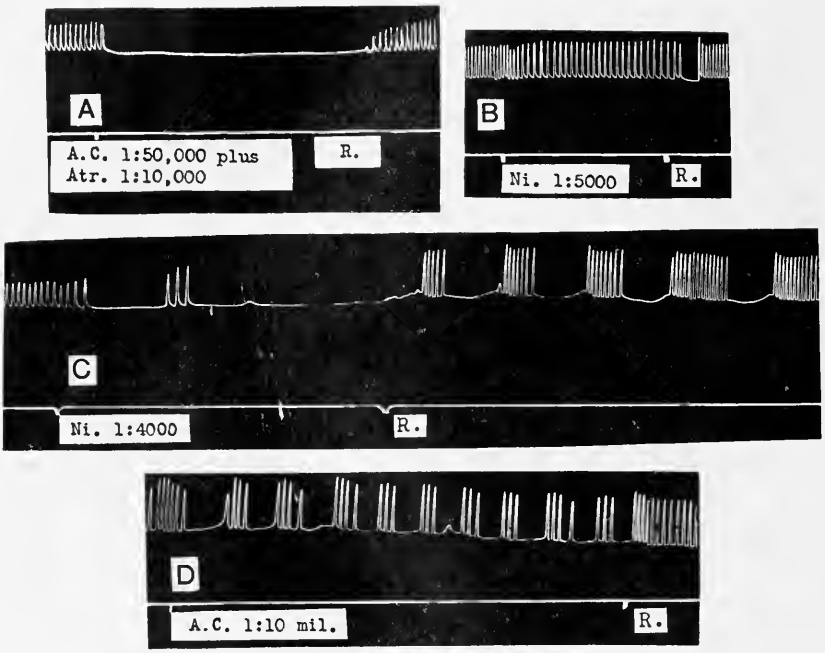

Fig. 2. *Ariolimax*. *A*, effect of acetylcholine 1 : 50,000 in mixture with atropine 1 : 10,000. *B*, effect of nicotine 1 : 5000. *C*, effect of nicotine 1 :4000. *D*, effect of acetylcholine 1 : 10,000,000 after treatment for 13 minutes with nicotine 1 : 5000.

was added an increase in magnitude of contraction (Fig. 2*B*). With nicotine 1 : 4000 diastolic stoppage with a few irregular beats of large magnitude occurred (Fig. 2*C*). The recovery from nicotine 1 : 4000 was interesting. After the nicotine solution was replaced by fresh Ringer, the heart beat five times with large amplitude and then stopped in diastole for a short time. This stoppage was followed by several strong beats, and that process of periodic stoppage, followed in each case

by a series of beats of increasing number, continued until the heart had resumed a normal though amplified rhythm. Nicotine 1 : 3000 caused complete diastolic stoppage, with recovery similar to that from nicotine 1 : 4000. Nicotine 1 : 1000 was toxic.

A heart was bathed for 13 minutes in 1 : 5000 solution of nicotine and maintained a slower though amplified beat; acetylcholine 1 : 10 million produced a periodic stoppage and resumption of beat (Fig. 2*D*). Acetylcholine 1 : 100,000 caused diastolic stoppage of a heart nicotinized with a 1 : 5000 solution.

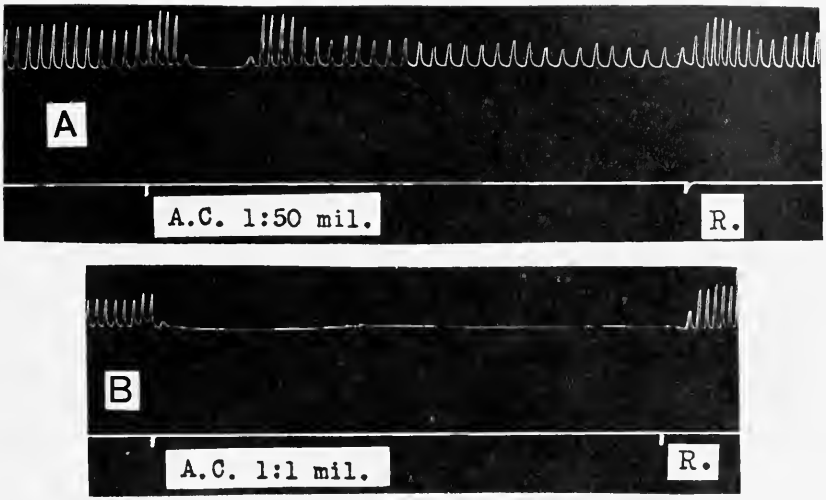

FIG. 3. *Ariolimax*. A, effect of acetylcholine 1 : 50,000,000 after treatment for 27 minutes with nicotine 1 : 8000. *B*, effect of acetylcholine 1 : 1,000,000 after treatment for 27 minutes with nicotine 1 : 8000.

After nicotinization of a heart with a 1 : 8000 nicotine solution for 27 minutes, acetylcholine effects were somewhat more characteristic. The most dilute acetylcholine solution to produce a noticeable effect was 1 : 500 million. This effect became more pronounced with progressively stronger solutions (Fig. 3*A*) until at acetylcholine 1 : 1 million diastolic stoppage occurred (Fig. 3*B*).

After treatment with nicotine 1 : 3000, which causes diastolic stoppage, a heart responded to local electrical stimulation with a strong contraction.

Conclusions

Acetylcholine apparently takes effect on the pacemaker mechanism of the heart, as Prosser (1940) found in *Venus*. This statement is con-

firmed by the fact that the *Ariolimax* heart, brought to diastolic stoppage by acetylcholine, reacts to direct local stimulation with a strong contraction; the contractile mechanism is therefore intact, but the pacemaker is impaired. Acetylcholine must also have an effect on the mechanism of contraction, for it produces a decrease in amplitude of beat; this may indicate a slight impairment of this mechanism. That this mechanism is not completely impaired is shown by the effect of electrical stimulation as cited above.

The effect of eserine in lowering the acetylcholine threshold indicates the existence of cholinesterase in the heart tissue.

Nicotine seems also to act upon the pacemaker mechanism, since at a low concentration it slows the heart; it also may affect the mechanism of contraction, since the amplitude is increased. At higher concentrations, it stops the beat with the ventricle in diastole; that this stoppage is not due to an impairment of the mechanism of contraction is shown by the fact that the heart reacts to direct electrical stimulation with a strong contraction. The phenomenon of grouped beats leading to the normal rhythm during recovery from nicotine treatment may be interpreted as the result of a gradual recovery of the pacemaker mechanism.

The experiments with acetylcholine on the nicotinized heart (Figs. 2*D*, 3*A*, 3*B*) present evidence that the two drugs have much the same effect on the pacemaker, though they apparently do not on the mechanism of contraction; concentrations of acetylcholine not high enough to affect the normal heart produce characteristic inhibitory effects on the nicotinized heart. The grouped beats observed in the nicotinized heart during treatment with acetylcholine 1 : 10 million (Fig. 2*D*) may therefore possibly be explained by assuming that the pacemaker mechanism, already affected by nicotine, was placed on the verge of cessation of activity (which would result in diastolic stoppage of the heart), a condition approximating that which existed during recovery from diastolic stoppage by nicotine 1 : 4000 (Fig. 2*C*).

One of the most fundamental observable differences in the response to drugs of molluscan and vertebrate hearts is the inability of atropine to block acetylcholine action in the former; this phenomenon was verified in *Ariolimax*.

Can it be said that the action of acetylcholine on the slug heart is "nicotine-like"? Atropine certainly fails to abolish its effects. The tests would seem to show that at least as far as the pacemaker is concerned, the effects of acetylcholine and nicotine are similar and additive. But the effects of the two drugs on the mechanism of contraction are certainly not the same.

Astacus trowbridgei

Brief preliminary tests were made on the heart of this animal with acetylcholine and other drugs. An attempt was made to corroborate the results obtained by MacLean and Beznak (1933), using the heart of *Astacus fluviatilis,* and to compare results with those obtained by Welsh testing the hearts of *Panulirus* and *Carcinus* (1939*a*, 1939*b*).

After the crayfish was pithed, a section of the carapace above the heart was removed and tissue surrounding the heart carefully trimmed away. Coagulation of the blood was prevented by immediate irrigation with physiological solution. A writing arm was then attached directly to the heart by means of a glass hook. Drug solutions were introduced directly into the cavity around the heart. By this technique a constant internal pressure was not maintained in the heart; however, in a number of tests results of considerable interest for comparison with those of other investigators were obtained. The heart of *Astacus* is delicate and sensitive to manipulation, but it is hoped that better records may be obtained by perfusion according to the technique of Cole et al. (1939) or by isolating the heart according to the technique of Welsh.

Drug solutions were made up from stock in Van Harreveld's solution: 12 grams NaCl, 1.5 grams $CaCl_2$, 14 grams KCl, .25 gram $MgCl_2$, (.29 gram $NaHCO_3$ per liter), plus distilled water to a liter.

All experiments conducted and solutions used were at room temperature (15°–20° C.).

Acetylcholine

There was found to be considerable individual variation in sensitivity to the mediator in these autoperfused hearts. However, acetylcholine 1:10,000 produced in a number of individuals a powerful increase in frequency and amplitude. In no tests with this concentration was there produced "a tonus increase . . . accompanied by a reduction in amplitude of beat due to incomplete recovery between strokes" or stoppage of the heart in systole (Welsh, 1939*a*).

Apparently MacLean and Beznak (1933) did not test concentrations of acetylcholine lower than 1:1,000,000. It was found that the heart of *Astacus trowbridgei* was insensitive to concentrations lower than 1:10,000,000,000 but at this concentration, which may be taken as the threshold, there was in a number of tests a noticeable increase in frequency. Welsh found that the isolated heart of *Carcinus* is insensitive to concentrations lower than 1:100,000,000 and that of *Panulirus* to concentrations lower than 1:10,000,000,000, which figure has been taken as the approximate threshold for the *Astacus* heart. A comparison of

these figures with the figures given for Mollusca in Table I will show that Prosser's (1940) statement based on his study of *Venus* that "the threshold for the accelerating action on arthropod hearts is higher than that for inhibition in the molluscan hearts" is not generally true.

Eserine

Welsh's tests with eserine on the isolated heart of *Panulirus* showed that with eserine 1 : 100,000 "if the heart had been beating in a somewhat irregular manner (e.g. exhibiting a series of grouped beats), a regular beat would be completely restored." This was found to be true also for the *Astacus* heart. If the heart had been beating regularly, administration of eserine often slightly increased the amplitude.

In a test during which eserine 1 : 100,000 was administered continuously the amplitude at first slightly increased, but later as the frequency also increased the amplitude of beat became less. With the inactivation of cholinesterase present in the heart one might expect somewhat the same results as produced by the administration of acetylcholine because of the sensitization of the heart to small amounts of the mediator that may be present.

Atropine

As has been shown for other arthropods, the stimulatory action of acetylcholine is abolished by atropine. In the crayfish tests showed that both amplitude and frequency effects of 1 : 10,000 acetylcholine are abolished by previous treatment with atropine 1 : 10,000.

Nicotine

The effect of nicotine on the *Astacus* heart seems similar to that on the heart of *Ariolimax*. In tests nicotine 1 : 10,000 produced a marked increase in amplitude and at the same time, if the heart was beating in Ringer with a swift regular beat, produced a slight decrease in frequency. At the higher concentration of 1 : 5000 which must approach toxicity, there occurred marked inhibition of both frequency and amplitude.

Adrenaline

Welsh showed that in *Panulirus* adrenaline in high concentrations had a powerful stimulatory effect of a different type from that of acetylcholine. In *Astacus* adrenaline 1 : 100,000 produces a violent acceleration and increase in amplitude and as in *Panulirus,* although the frequency rapidly returns to normal, the amplitude effect remained for some time after replacement by Ringer.

Although MacLean and Beznak (1933) say that in *Astacus fluviatilis* they were able to produce only a slight increase in frequency with adrenaline 1 : 1000, in *Astacus trowbridgei* concentrations of 1 : 1,000,000 produce a sharp increase in frequency without changing the amplitude.

Conclusions

Can it be said that the action of acetylcholine on the crayfish heart is "muscarine-like"? Atropine abolishes its effects. As has been stated, the effects of nicotine on this heart do not resemble the effects of acetylcholine on it, but they are remarkably similar to the effects produced by nicotine on the slug heart.

It is too early to conclude whether or not these two actions of acetylcholine, the "nicotine-like" and the "muscarine-like" well known in the vertebrates, really are found among the invertebrates. It is hoped that future experiments may cast light on this problem.

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